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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXVI | *OCTOBER, 1947* | NUMBER 10 | CONSECUTIVE NO. 20

An *In Vitro* and *In Vivo* Study of Glass Particles in Ampules*,†

By JOHN H. BREWER and J. H. FITZGERALD DUNNING‡

This report on the effects of glass particles when injected into animals indicates that massive doses are required to produce damage to the organs examined during the study.

IN AN EFFORT to improve the quality of ampule preparations, studies have been made from time to time to determine the nature of contaminants found in ampules discarded after routine manufacturing inspection. It was ascertained that many of these ampules were discarded because of the presence of glass particles which range in size from less than one micron to those visible to the unaided eye. In view of the fact that many physicians are of the opinion that a glass particle injected intravenously would serve as an embolus, regardless of size, the present study has been undertaken. The investigation has also been stimulated by the development of electronic instruments which can pick up particles beyond the scope of the unaided eye. It was thought important, therefore, to determine the significance of these particles, for it has been observed that it is practically impossible to prepare ampule solutions in glass and to sterilize by autoclaving without producing some glass contamination. From a common sense viewpoint, it was believed advisable to determine at what level glass contamination in ampule solutions might be expected to be harmful. On this basis several experiments were planned which we felt might give some practical results.

Solutions containing various sizes of glass particles were prepared by the method described in the *Journal of American Public Health*, Vol. 29, p. 1147, October, 1939. These suspensions of glass particles have been injected intravenously in animals in varying amounts and over time periods extending to almost one year. These animals have been sacrificed, and chemical, histological, and pathological studies have been made. In addition, more than one thousand animals have been injected with concentrated residues obtained from ampules rejected because of particle contamination covering a period of some months of manufacture.

The object in making this study is, of

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† This is primarily the intravenous study of this problem. A later paper will deal with carbonized particles, fiber, and intramuscular injection of these materials.

‡ The authors are indebted to Mr. A. E. Stickels for the chemical recovery of the glass particles, and to Dr. John Slaughter for the pathological sections. We also wish to thank Mr. T. J. Carski for the photomicrographs.

course, not to promote the use of ampules containing visible particles, but is an attempt to add to the rather meager information on the subject.

Medical literature abounds in discussions of silica and silicosis, the presence of silica in blood, urine and tissues, but most of this literature deals with injected or respired silica and its compounds. One is reminded of an incident in the life of Sir Harry Lauder, when he was approached at the stage door by a woman from the Salvation Army and asked to give until it hurt, replied, "Lady, the very thought of it hurts." It would seem that the very thought of injecting glass intravenously has been generally avoided in making such studies. However, in a series of papers on the biochemistry of silicic acid, King, Stantial, and Dolan, in 1933 (1) describe the intravenous administration of particulate silica in dogs. They gave 250 cc. of a SiO_2 suspension containing 0.8 mg. per cc. This was given over a period of three hours and at six hours the dog died. No information was given of particle size. Gardner and Cummings (2), in 1931, Szurek and Czaja (3) in 1933, and Simson (4, 5) in 1937 and 1940 report other experiments on the intravenous injection of particulate silica or glass. Although these papers were studies on various phases of silicosis, some of them do give particle size and their findings were helpful to us in a study of this problem. For the most part, these papers discuss the injection of particles of less than 2 microns in diameter and Simson states that 98 per cent of his particles were 0.4 micron, or less. The results of these experiments led Simson to state that at autopsy the organs and tissues of the animals showed no macroscopic evidence of disease. Microscopically, the lungs and kidneys showed numerous capillary emboli composed of aggregates of silicotic particles which later disappeared. In summarizing his experimental results, Simson stated that distinctive fibrotic lesions are produced in the liver spleen and lymph nodes of rabbits following intravenous injection of silicious dust of 0.8 micron, or less, in size.

Gardner and Cummings summarize their results as follows: Three series of rabbits were injected intravenously with 1.3 Gm., respectively, of silica particles 1 to 3 microns in diameter and silica particles 6 to 12 microns in diameter. The injections were given in divided doses and required from one to four months for their completion.

These particles were segregated in different locations according to their size. The largest ones were caught in the pulmonary capillaries, those of intermediate size in the spleen and hepatic lymph node, and the finest ones in the liver.

Fine silica particles are most active and have produced a progressive, coarsely nodular cirrhosis of the liver attended by extensive destruction of the parenchyma, followed later by regeneration in certain areas. This cirrhosis is the result of a typical, hyaline, nodular, silicotic fibrosis originating in the portal connective tissues.

Coarse silica particles, 10 to 12 microns in diameter, are much less irritating. They excite a simple foreign body type of reaction which has progressed very little in practically three years' time.

The importance of the factor of particle size in producing reaction to silica is the chief contribution of this experiment. It has been shown that large particles, 10 to 12 microns in diameter, provoke a foreign body reaction in the lungs which persists without much further change for nearly three years. Smaller ones, from 3 to 6 microns in diameter, cause slowly progressive changes in the spleen and hepatic lymph node. Very fine ones of the order of 1 micron in size result in progressive proliferation of connective tissue in the liver.

EXPERIMENTAL

In our own experimental work, we thought it important to determine what size glass particles would be encountered in ampule preparations and what size particles could be picked up by ordinary examination by a person whose vision was approximately 20–20. Using a 100-watt lamp and examining against a black and white background, the various preparations were studied. Consultation with ophthalmologists and a literature survey gave us the impression that the human eye should be able to see particles of about 50 microns under such conditions. We were surprised, however, to find that our ampule inspectors were discarding ampules containing particles no larger than 1 or 2 microns. This is

entirely understandable if one recalls having seen the reflection of an automobile windshield on a mountain so far away that the car itself could not be seen.

On examining a large number of ampules from several sources, both autoclaved and hot-air sterilized, we found most of the glass particles to be larger than 1 micron, with an average size of about 20 microns; some were as much as 75 microns in length but only a few microns in diameter. Since it is entirely possible for such particles to pass a 20-gauge needle, experimental work was planned so as to study the effect of injecting intravenously into animals suspensions containing particles in this range of size. Some animals were injected with suspensions of fine glass only; others received injections of coarser particles in both light and heavy suspension. Suspensions were made of mixtures of soft glass and of borosilicate glass, and some suspensions were made of pyrex glass only.

To describe briefly the actual technique of animal experimentation, rabbits received, by ear vein injection, varying quantities of different suspensions of glass particles. For example, one group of animals was given daily injections so that they received in thirty-two days a total of 0.416 Gm. of glass per animal. Based on body weight, this would be about 14 Gm. in man in one month, or about 0.5 Gm. per day. Since this is an absurd amount of glass to expect in ampule contamination, other animals have been injected over longer periods of time with lighter suspensions, 0.0025 Gm. per cc. for seventy-six days, and no pathology was revealed. However, an occasional particle of glass was found in the lungs.

One animal received a total of 670.5 cc. of glass suspension containing 4.156 Gm. of glass. The average daily dose was 0.0124 Gm. and the largest single injection was 0.085 Gm. This was given in 153 injections over 334 days and the animal which was seemingly well was killed and examined. In the animal which had been injected for 334 days with an average daily dose of 0.0124 Gm., macroscopically the organs revealed an enlarged liver and spleen but the other organs appeared normal. Microscopically there was a generalized picture of silicosis.

Since the smallest capillaries in the body are about 8 microns in diameter, as compared with the red cells at about 4.5 microns, we can rationalize as to what happens when glass particles as described are injected intravenously. The venous blood first encounters these small capillaries in the lungs when being aerated. As might be expected, there is some plugging of the capillaries with the larger pieces but one gets more of a Beaver Dam effect due to the sharp shape of the glass and the fluid nature of the cells; they tend to flow around the glass. When 1 cc. of a 1.3% suspension of this glass is injected daily for thirty-two days, there is no pneumonia or inflammation. The venules are dilated to capacity. The capillaries are moderately engorged. Some of the arterioles are thick walled. There is variation in the degree of aeriation atelectasis. There is poor circulation in patches. Many capillaries are bulged, widening the septiae, and these capillaries contain ragged shaped crystals, some of which are embedded in a bluish homogeneous fluid-like substance in which there are endothelial cells and leucocytes, suggesting early organization of thrombi. There are still clear spaces indicating that capillary luminae are not completely obliterated in spite of the bulging. However, there are crystals outside of the capillaries but this may be attributed to mechanical factors, since the microtome knife could not cut the particles of glass and simply shoved them along into the alveolar spaces. The smaller particles over the short time period of thirty-two days are carried along and are apparently disposed of without producing pathology, since none of the other organs are seemingly involved. Grossly the animals appeared well and had no dietary lay-off. Macroscopically the organs appeared normal and even the lungs had no gross pathology.

In the lungs of the one-year rabbit there are large discrete tubercle-like lesions which have three types of zones: (*a*) The outer lymphocytes, (*b*) Middle large cells with clear oval-shaped nuclei resembling an epitheloid pattern, and (*c*) The core is composed of polymorphonuclear leucocytes and necrotic substances. These lesions are quite large and resemble focal lesions encountered in silicosis. The capillaries are made conspicuous by small needle-shaped crystals. The alveolar septae are usually broad and practically all alveolar spaces present knob-like structures which are part of the septae bulging into alveolar space. The luminae of these circular, sharp, definite knobs are connected with the capillary wall and contain fragments of highly refractory crystalline substance. Many of these are completely occluded by large mono- and multi-nucleated cells. The multi-nucleated cells are interpreted as foreign body giant cells. There is no leucocytic infiltration into the parenchyma beyond the capillary saculations. The larger arterioles are thick walled and the capillary wall is moderately engorged and has some edema.

In the liver small glass particles are seen. The liver parenchyma is not distorted. The lobules maintain their normal pattern. The cytoplasmic cells have a coarse, flaky texture but no distinct vacuoles are seen. There is no necrosis or leucocytic reaction except in the protal triads. Here the cells consist of small lymphocytes and large multi-nucleated giant cells. The cytoplasm of these giant cells contains fine crystal and needle-like refractile substances; also, a granular matter of the same appearance. These areas of granulation are so well defined and separated from the parenchyma and septae as to be interpreted as over-stretched vessel walls. There are also multi-nucleated giant cells along the triads which cannot be localized as to inside or outside of the vessels. There are many nests of multi-nucleated giant cells; also, single giant cells scattered throughout the parenchyma, all distinctly replacing liver cords and appear to rest free in the sinusoids. All of these contain particles of refractile bodies. The striking feature of this picture is that these giant

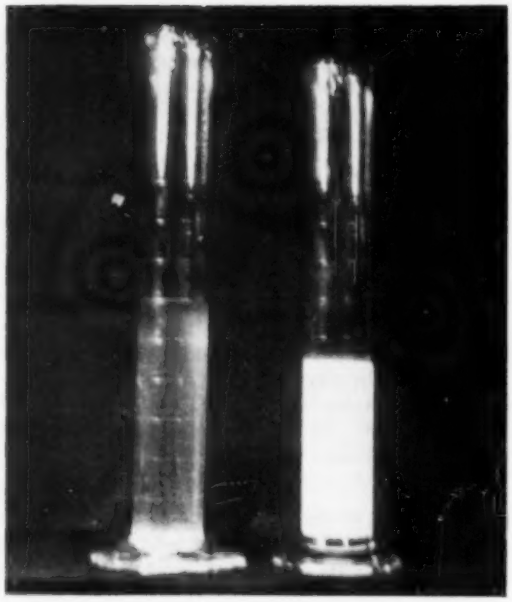


Fig. 1.—Graduate on left contains light suspension of glass. Graduate on right contains heavy suspension.

(Photomicrographs represented in Figs. 2–6 are all of the same magnification.)

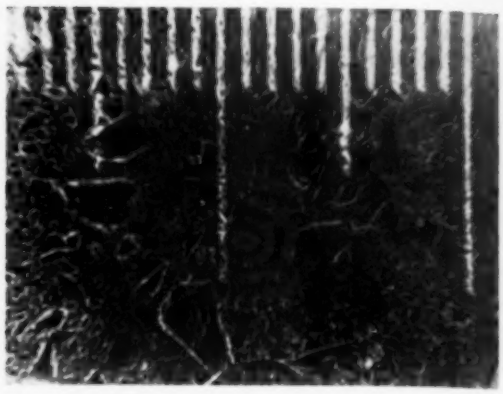
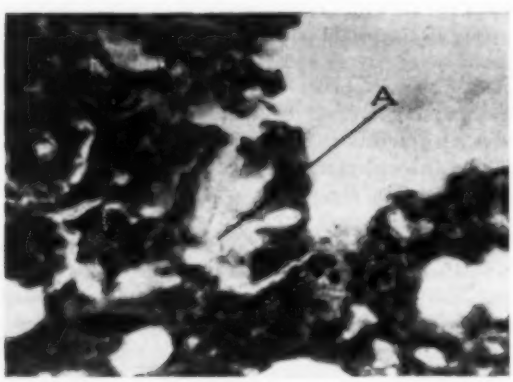
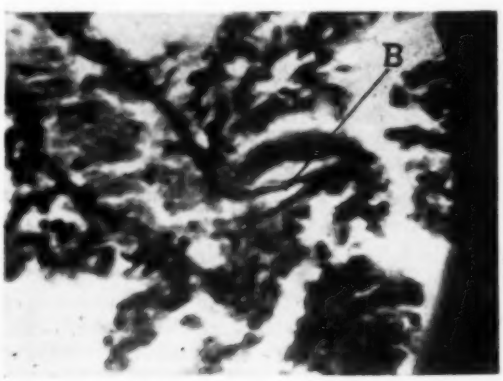


Fig. 2.—Photomicrograph of heavy glass suspension; the smallest divisions on above scale are 10 microns.

Fig. 3.—Glass particles in light suspension scale same as Fig. 2. These particles have passed through hard filter paper.

Fig. 4.—These glass particles were recovered from the lungs of a rabbit injected for 30 days with the solution shown in Fig. 2.





Figs. 5 and 6.—Show glass particles in the lungs. "A" shows that there is not a complete blocking of the capillary by the particles, and "B" shows 2 sharp glass particles lodged in the walls of a capillary.

cells are bare, thus, not producing any destruction of adjacent parenchyma nor stimulating the leucocytic zones around them. They may be Kupffler's cells. There is no cloudy swelling; the appearance may be due to some change in the feeding habit of the animal.

Spleen.—The parenchyma is studded with giant cells which have a tendency to nest and produce no reaction.

Intestine.—Fine glass particles found.

Kidney.—Occasional giant cells are found and the glomeruli are gorged with blood.

In another series of animals, in which the glass was very dilute to simulate more closely actual ampule contamination and which had been filtered to remove the larger particles, no glass was found in the lungs and no pathology was encountered. All sections looked normal and these sections have been studied by consulting pathologists who confirmed these findings.

DISCUSSION

It is interesting to note that chemical examinations were confirmatory of the histological and pathological findings in those animals which received the 1.3 per cent glass for thirty-two days. The lungs, which contained large size glass particles, were digested with acid and assay revealed that, calculated on the wet weight of lungs, there was present about 1 per cent glass. In the animal which received a lighter suspension for nearly a year almost 2 per cent glass was found. There was one very important difference. The silica in the rabbits which had received the glass for only a short time gave about the same loss with hydrofluoric acid as the control glass suspension. The animal which had had the glass for almost a year gave a 95.15 per cent loss, indicating that the body had dissolved out the basic ion, leaving silicic acid which would account for the tissue damage and generalized picture of silicosis. These findings are typical of animals injected for one year. Chemically, no glass was found in those organs in the animals which received glass over shorter periods of time except the lungs in those animals as described.

From a practical point of view, 1,089 mice were injected intravenously with the centrifugate obtained from ampules regularly processed and discarded because of particle contamination. This was continued over a period of several months to attempt to produce fatal embolism with these particles. The particles were for the most part glass, but some were charred material, probably carbon, while others were fibers. In no case did death occur, and no latent effects were noticed.

Although the industrial hygienist and those who come in constant contact with ultra small glass particles must be constantly on the alert for pathology connected with silica injection, the ampule manufacturer who meets the present requirements of producing ampules substantially free from glass particles which can be seen with the unaided eye need have no fear that his product on injection would cause a fatal embolism, even though an occasional particle falls into the solution on breaking open the ampule. Furthermore, repeated injection of occasional particles will not give rise to pathology which could be considered dangerous. We must be warned, however, against processes of manufacture such as grinding, colloidizing, or rapid temperature shock which might produce large quantities of ultra fine glass of less than 1 micron without instituting filtration procedures for removing it. Simple filtration through hard paper is not sufficient to remove such particles. Several types of filters satisfactory for bacterial removal are efficient for this purpose and should be used, although the material is subsequently sterilized by other means.

SUMMARY AND CONCLUSION

In this investigation, without attempting to be exhaustive, we have tried to prove that occasional particle contamination of ampule preparations produces no significant pathology in animals. It has been shown that truly massive doses are required to produce any pathological reaction in rabbits.

It can be reasoned, therefore, that by careful ampule processing, the dangers inherent in particle contamination can be largely eliminated.

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Microscopic-Crystallographic Properties of Crystalline Sodium Penicillin G*

By WILLIAM V. EISENBERG and GEORGE L. KEENAN

FOR THE purpose of establishing microscopic criteria that would be useful as physical data for characterizing the sodium salt of crystalline penicillin, factor G, an optical-crystallographic study was undertaken of five preparations of this substance obtained from different sources. Four samples were produced commercially while the fifth was obtained from a government research laboratory. These samples were studied by means of the immersion method as applied to the examination of crystalline material, which consists of mounting the material in oily liquids of known refractive index and making observations in ordinary light, and parallel and convergent polarized light with the polarizing microscope.

The microscopic examination of the five preparations in ordinary light at a magnification of approximately 200× revealed three crystalline habits. Elongated, fibrous rods, apparently aggregates of needle-like crystals, characterized two of these samples (Figs. 1 and 2). Two samples consisted of plates, in one case more elongated (Fig. 3) than in the other (Fig, 4), and compact radial aggregates (spherulites) represented the third habit (Fig. 5).

From a microscopic-crystallographic study of these five samples three significant refractive indices were determined, $n\alpha = 1.550$, $n\beta = 1.609$, $n\gamma = 1.620$, all ± 0.002.[1] The most common index for determinative purposes was found to be $n\beta$, shown on plates exhibiting partial extinction and optic axis figures. This index is most readily shown where the material crystallizes largely as plates (Fig. 3 and 4). It may also be measured on minute rods and needles occurring in the other habits (Figs. 1, 2, and 5). $n\alpha$ and $n\gamma$ are not as frequently shown as $n\beta$.

In parallel polarized light (crossed nicols) samples crystallizing in elongated fibrous rods (Figs. 1 and 2) showed parallel extinction and positive elongation. In convergent polarized light (crossed nicols), the usual interference figure shown was that of the optic axis. Platy material such as that illustrated by Fig. 3 showed that the material was biaxial negative with large axial angle as may be determined occasionally on interference figures showing curved isogyres.

Although direct examination of the salts in ordinary light showed that they exhibited various habits, this was apparently due to different methods of preparation or crystallization, and all of the samples could be converted to the same habit by recrystallizing a small amount from a drop of 95% ethyl alcohol on a microscopic slide. The material to be recrystallized was treated with the alcohol and the preparation vigorously stirred with a glass rod to induce crystallization by reseeding with the material that had not completely dissolved. The addition of *n*-butanol to the alcohol solution will aid the crystallization. This treatment of the crystalline preparations caused the substance to crystallize as plates having a 6-sided outline, many of which develop in hemimorphic formations (Fig. 6).

Heating all five of these preparations at a temperature of 100° for two hours produced no change in the crystalline habit or in the optical-crystallographic properties. It is obvious from this that crystalline penicillin sodium contains no solvent of crystallization. The five compounds of crystalline penicillin sodium studied are identical with respect to their optical-crystallographic properties and these may be used for establishing the identity of this substance.

Reproductions of photomicrographs showing the crystal structures of the samples used in this investigation are shown on the opposite page.

* Received Jan. 30, 1947, from the Microbiological Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.

[1] Refractive indices are given for the D-line (Na) at 20°.

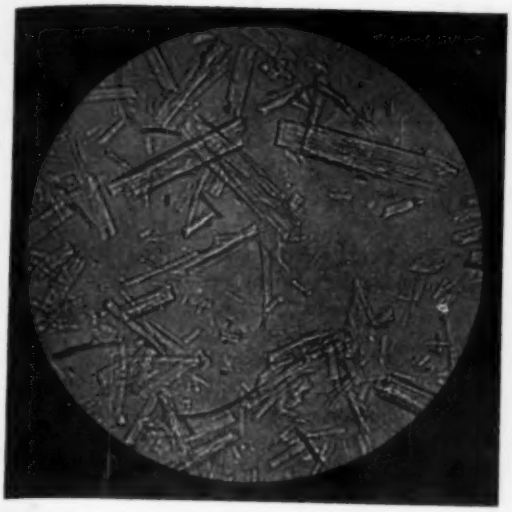

Figure 1 (150×).

Figure 2 (150×).

Figure 3 (150×).

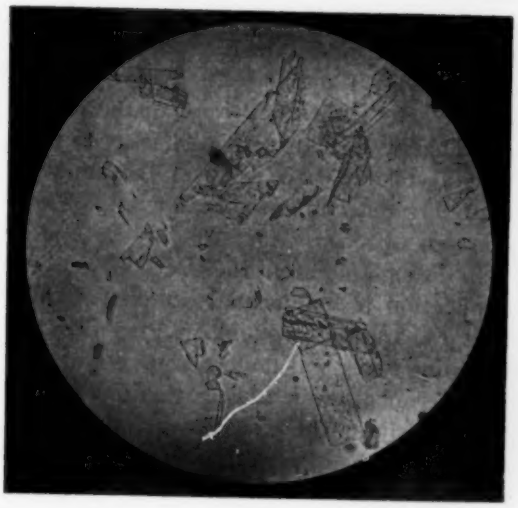

Figure 4 (60×).

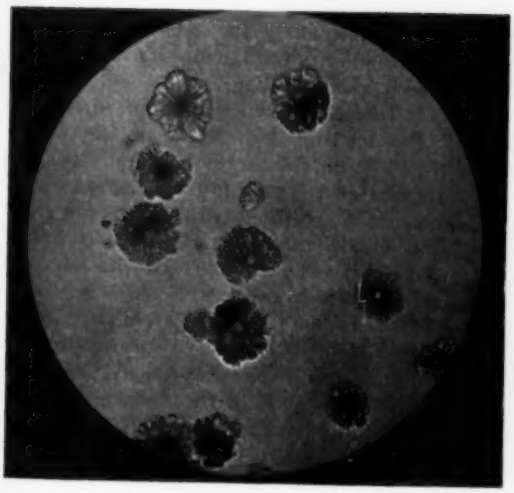

Figure 5 (150×).

Figure 6 (150×)

A Study of the Modified Knudson–Dresbach Colorimetric Evaluation of Digitalis: Extraction Studies on Digitalis*

By SAMUEL W. GOLDSTEIN†,‡

The Knudson–Dresbach colorimetric method as modified by Bell and Krantz for the assay of digitalis preparations has been applied to several samples. The results of this study are reported.

BECAUSE NONE of the present biological assay methods for digitalis is entirely satisfactory, Bell and Krantz studied the Knudson and Dresbach (1) colorimetric determination based on the Baljet (2) reaction between alkaline picrate solution and digitalis glycosides. In 1945 they published their modified method (3). This method gave results sufficiently close to the results obtained by the cat method of assay to warrant a collaborative study of the assay of digitalis and its preparations by both methods (4).

In order to check the method and its application to commercial samples of digitalis preparations available in drugstores the following study was undertaken.

Experiments were also carried out to test the necessity of the U. S. P. requirement of a twenty-four-hour period of extraction in a mechanical shaker for digitalis and preparations containing powdered digitalis.

EXPERIMENTAL

A "facile" test bottle shaker (Wagner Glass Works, New York) was used and the powdered samples and menstruum (alcohol 4 volumes, water 1 volume) were placed in oil sample bottles which fitted into the shaker cups. A Fisher Electrophotometer with green filter No. 525 and 23-cc. absorption tubes was used. All readings were made on the logarithmic scale of the instrument. The assay procedure was carried out exactly as described by Bell and Krantz (3). Readings were made at ten-minute intervals over a period of sixty minutes.

Digitalis Tinctures.—Data obtained from twenty determinations with six Reference Standard Tinctures and a number of commercial tinctures are represented graphically in Fig. 1 by plotting the readings (logarithm of the transmission) against time intervals.

The curves obtained with the U. S. P. Reference Standard Tinctures and half-strength Reference Standard Tinctures are practically identical with those reported by Bell and Krantz (3). The curves obtained with commercial tinctures assaying near or below 100% at the twenty-minute interval follow fairly closely the curves obtained with the Reference Standard Tinctures. But the curves obtained with commercial tinctures assaying considerably above 100% at the twenty-minute interval show a wide divergence from the standard curves. The curve showing the highest values was obtained from results with two samples from manufacturer No. 5, and one other sample. The drugstore operator could not estimate the age of this latter preparation, and it might be possible that he assigned it to the wrong manufacturer. The next highest curve represents another sample from manufacturer No. 5.

Bell and Krantz (5), as a result of their studies with the digitalis glycosides, have assumed that the intensity of the Baljet reaction is proportional to the amount of glycoside (lactone) present, but that the degree of isomerization [which occurs in the presence of the Baljet reagent] is not directly proportional to the concentration of the glycoside. They conclude that if the observed intensity of the Baljet reaction is regarded as the resultant of these two factors, and since one of them does not show direct proportionality to the concentration, the resultant likewise will not. Their assumptions and conclusions are definitely applicable to the results obtained with most of the tinctures of widely varying potencies as shown in Fig. 1.

Digitalis Tablets.—Tinctures were prepared from eleven commercial digitalis tablets using the U. S. P. XIII procedure for the assay of Digitalis Tablets. These tinctures were tested in the same manner as the commercial tinctures, and the results are given graphically in Fig. 2. The curves obtained follow the same general pattern as observed with the commercial tinctures, with two exceptions. These samples show an unusual divergence from the general curve and undoubtedly contained some substance, possibly lactose, which gives a strongly increasing color reaction with the Baljet reagent.

The results from both the tinctures and the tablets show that where there is evident divergence from the standard curves, this divergence increases as the time interval increases. It may be assumed

* Received March 15, 1947, from the State of Maryland Department of Health, Baltimore.
† Pharmaceutical Chemist, State of Maryland Department of Health.
‡ The author is indebted to Mr. J. R. McComas for assistance with the charts.

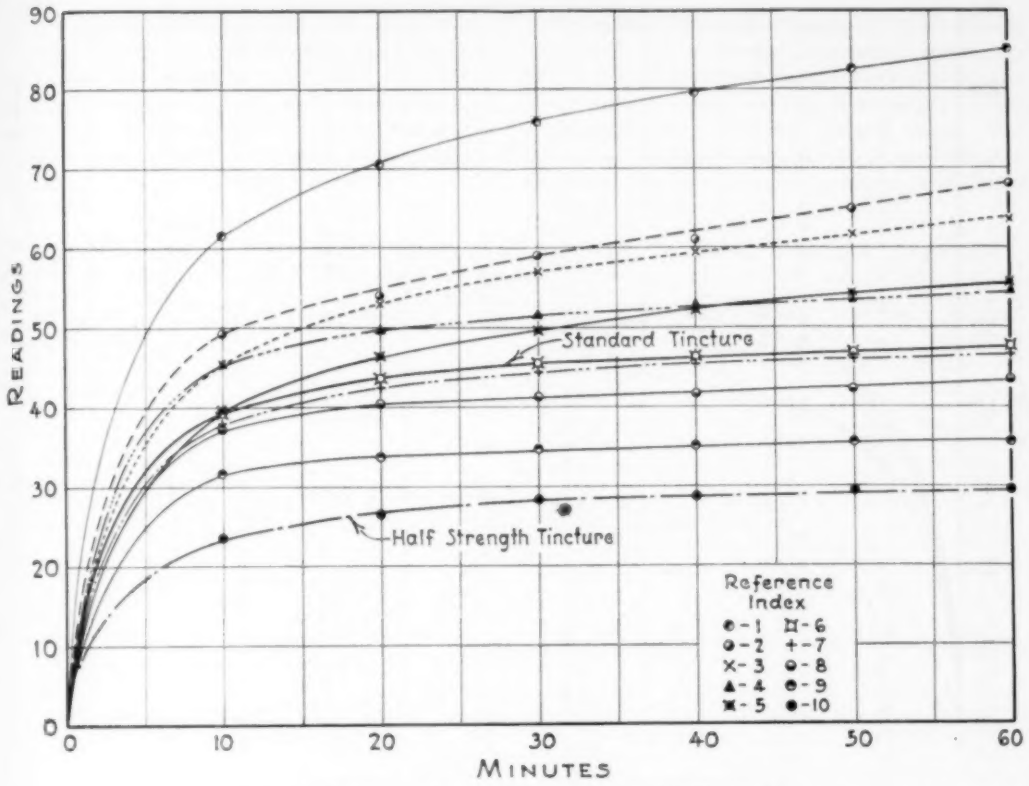

Fig. 1.—Reference Standard and Commercial Tinctures

Curve No. 1. Average values for samples 17283 S, 17287 S, and 8922 E, which assayed 180% ± 4 at 20 minutes.
Curve No. 2. Sample 17293 S, which assayed 128% at 20 minutes.
Curve No. 3. Average values for samples 17278 S and 17315 S, which assayed 130% ± 3 at 20 minutes.
Curve No. 4. Average values for samples 17280 S, 17292 S, and 8925 E, which assayed 117% ± 2 at 20 minutes.
Curve No. 5. Average values for samples 17279 S, 17286 S, and 17288 S, which assayed 109% ± 3 at 20 minutes.
Curve No. 6. Reference Standard Tincture.
Curve No. 7. Average values for samples 17276 S, 17281 S, and 17282 S, which assayed 100% ± 2 at 20 minutes.
Curve No. 8. Average values for samples 17289 S, 17290 S, 8923 E, and 8924 E, which assayed 90% ± 2 at 20 minutes.
Curve No. 9. Sample 17291 S, which assayed 68% at 20 minutes.
Curve No. 10. Half-strength Reference Standard Tincture.

that the earliest consistently reproducible reading would be more likely to give results based on differences in concentration of the glycosides in *D. purpurea* and its preparations. This happens to be the reading taken about twenty minutes after the addition of the reagent to the prepared sample. Bell and Krantz have selected this time interval. Published results (3, 5, 6) indicate that values obtained by the modified Knudson–Dresbach chemical assay are generally higher than the values obtained by bioassay procedures. These differences would be further accentuated if values were based on readings taken after an interval longer than twenty minutes, especially with preparations considerably stronger than the standard tincture. This purely factual consideration is sufficient to warrant the selection of the twenty-minute reaction period.

The assay results for the commercial tinctures at the different time intervals, together with the approximate length of time the tinctures were known to have been in the drugstores, are given in Table II. It is noteworthy that of the identified tinctures the highest percentages were obtained with old products of manufacturer No. 5. The comparatively fresh product of the same manufacturer showed an increase of 33% between the twenty-minute and sixty-minute determinations. The next greatest increase in percentage between the twenty-minute and sixty-minute determinations is 18%. Apparently some substance is present in the No. 5 preparations (possibly introduced in the menstruum) which slowly changes or causes a change under ordinary conditions with the change being accelerated in the presence of the alkaline picrate reagent. Tinctures

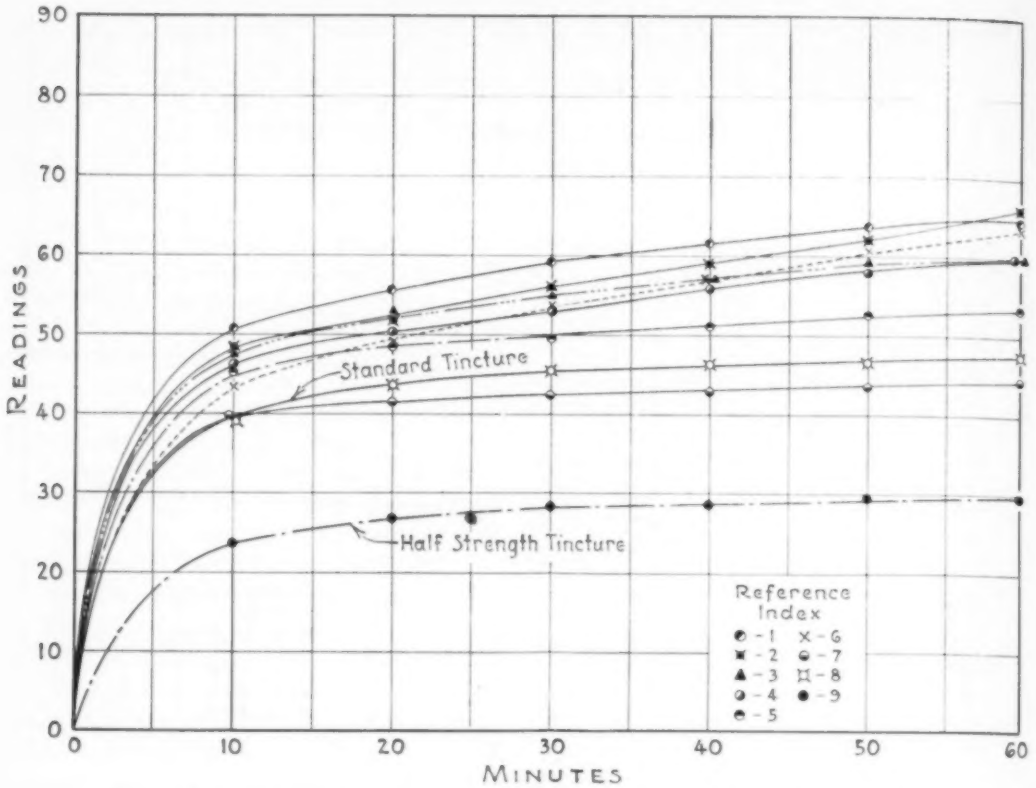

Fig. 2.—Reference Standard Tinctures and Tinctures Prepared from Digitalis Tablets

Curve No. 1. Average values for samples 17256 S and 17265 S, which assayed 135% ± 2 at 20 minutes.
Curve No. 2. Sample 17260 S, which assayed 124% at 20 minutes.
Curve No. 3. Average values for samples 17264 S and 17266 S, which assayed 128% at 20 minutes.
Curve No. 4. Sample 17259 S, which assayed 122% at 20 minutes.
Curve No. 5. Sample 17261 S, which assayed 113% at 20 minutes.
Curve No. 6. Sample 17258 S, which assayed 116% at 20 minutes.
Curve No. 7. Average values for samples 17257 S, 17262 S, and 17263 S, which assayed 95% ± 5 at 20 minutes.
Curve No. 8. Reference Standard Tincture.
Curve No. 9. Half-strength Reference Standard Tincture.

giving such results should be checked by biological assay.

Omitting from consideration the established products of manufacturer No. 5, fifteen of the twenty-three tinctures fall within the limits allowed by the U. S. P. The age of sample 17283 S could not be established, and it appears doubtful that it was assigned to the correct manufacturer.

Of the eleven samples of Digitalis Tablets tested, four samples exceeded the U. S. P. limit in potency. However the amounts by which the upper limit was exceeded were 2%, 2%, 7%, and 10%. These four samples were products of the same manufacturer, and their curves indicate the absence of any interfering substance.

Extraction Studies.—Digitalis tinctures were prepared from U. S. P. Standard Reference Powder by the official procedure except that eight-, sixteen-, and twenty-four hour extraction periods in a mechanical shaker were used. The averages of values for three, twelve, and five series of readings of the respective products are given in Table I. The values obtained indicate that for all practical purposes an extraction period of eight hours in a mechanical shaker allows complete extraction of the glycosides of *D. purpurea*.

TABLE I.—LOGARITHMIC SCALE READINGS FOR TINCTURES PREPARED FROM REFERENCE STANDARD DIGITALIS USING DIFFERENT EXTRACTION PERIODS

Reading Time, Min.	8-Hr. S[a]	8-Hr. S/2[b]	16-Hr. S	16-Hr. S/2	24-Hr. S	24-Hr. S/2
10	39.8	23.5	39.3	23.5	40.0	23.8
20	43.7	26.5	43.3	26.7	43.9	27.1
30	45.3	27.5	45.3	28.0	46.0	28.6
40	46.0	28.0	46.0	28.7	46.5	29.3
50	46.5	28.5	46.6	29.1	47.2	29.6
60	47.0	28.7	47.1	29.3	47.3	30.1

[a] S is the full-strength Reference Standard Tincture.
[b] S/2 is the half-strength Reference Standard Tincture.

Tinctures were prepared from commercial Digitalis Tablets by the U. S. P. XIII procedure and also

by limiting the extraction period to eight hours. The results of the assays of the tinctures obtained by both procedures are given in Table III. These results appear to establish beyond a doubt that an eight-hour extraction period in a mechanical shaker is sufficient for complete extraction of the digitalis glycosides.

Discussion of Analytical Method.—In its present state the Knudson-Dresbach method as modified by Bell and Krantz for the evaluation of digitalis and its preparations may not appear to satisfy all the requirements for an official chemical assay. But neither is the official biological method entirely satisfactory. The chemical procedure does yield results

TABLE II.—PER CENT POTENCY IN TERMS OF STANDARD OF COMMERCIAL DIGITALIS TINCTURES

Sample No.	Mfr. No.	Time in Store	% at 10 Min.	% at 20 Min.	% at 30 Min.	% at 40 Min.	% at 50 Min.	% at 60 Min.
17273 S	1	1 wk.	...	95	...	...	...	...
17274 S	2	3 mo.	...	126	...	...	...	...
17275 S	1	5 mo.	...	97	...	...	...	...
17276 S	3	2 yr.	...	102	100	100	100	100
17277 S	3	1 yr.	...	120	123	124	126	126
17278 S	..	....	...	132	134	141	143	146
17279 S	3	2 mo.	...	111	110	113	117	121
17280 S	4	6 mo.	120	115	111	111	113	113
17281 S	..	4 mo.	...	102	100	100	100	100
17282 S	1	2 mo.	100	98	96	96	96	96
17283 S	3	....	161	181	187	196	197	207
17284 S	4	2 yr.	139	143	141	144	147	149
17285 S	2	2 mo.	121	138	141	147	150	154
17286 S	2	10 mo.	102	106	114	120	123	124
17287 S	5	18 mo.	173	178	186	195	194	199
17288 S	2	1 yr.	103	108	114	120	120	121
17289 S	1	1 mo.	90	90	89	87	87	89
17290 S	1	....	94	91	89	86	86	87
17291 S	1	4 mo.	72	68	67	65	65	64
17292 S	2	6 mo.	106	118	125	127	128	129
17293 S	5	2 mo.	127	130	140	144	153	163
17315 S	2	2 mo.	116	127	133	136	141	143
8922 E	5	7 mo.	182	184	191	200	207	210
8923 E	..	7 mo.	97	92	90	91	90	89
8924 E	1	2 wk.	93	88	85	85	84	83
8925 E	4	6 mo.	121	119	114	116	117	114

TABLE III.—PER CENT POTENCY IN TERMS OF STANDARD DIGITALIS TABLETS
Samples Prepared by Shaking for 8-Hour and for 24-Hour Periods

Sample No.		Instrument Reading Intervals in Minutes 10	20	30	40	50	60
17256 S,[a]	8 hr.	139	139	141	149	153	154
	24 hr.	133	133	139	143	150	151
17257 S,	8 hr.	95	91	88	90	90	90
	24 hr.	95	89	88	91	91	93
17258 S,	8 hr.	110	114	122	128	138	143
	24 hr.	113	117	124	134	141	146
17259 S,	8 hr.	126	124	126	133	137	140
	24 hr.	121	120	120	127	131	135
17260 S,[b]	8 hr.	127	124	130	137	144	151
	24 hr.	125	124	130	137	144	151
17261 S,	8 hr.	120	112	110	115	116	115
	24 hr.	120	114	112	115	119	118
17262 S,[c]	8 hr.	113	100	96	97	99	99
	24 hr.	109	100	96	94	96	97
17263 S,	8 hr.	98	94	91	88	87	87
	24 hr.	102	97	93	90	90	90
17264 S,[a]	8 hr.	127	128	132	134	139	140
	24 hr.	127	128	132	134	139	140
17265 S,[a]	8 hr.	140	133	136	142	146	147
	24 hr.	137	133	136	142	146	147
17266 S,[a]	8 hr.	124	126	126	130	131	130
	24 hr.	132	129	131	136	140	138

[a] Samples, all from the same manufacturer, yielded green filtrates.
[b] Green colored, coated tablets yielded green filtrates.
[c] The 8-hour preparation was tested 5 days earlier than the 24-hour preparation. The prevailing room temperatures were 21° and 19.5°, respectively.

that check very well, and earlier studies (4) have indicated that both methods are measuring the same activity of the drug. The chemical procedure certainly can be useful in control laboratories that are not equipped for biological procedures. As a precaution, the final readings can be continued at ten-minute intervals beyond the critical twenty-minute period, and the curve, obtained by plotting the readings against time, can be compared with curves prepared by assaying biologically standardized samples. When a curve is definitely out of line, indicating the presence of an interfering substance, that sample can be sent to another laboratory for checking by a biological procedure.

Inspector H. Bernhardt collected the commercial samples from drugstores and obtained information, wherever possible, as to the length of time the tinctures had been in stock. This did not take into account the age of the preparations at the time the pharmacists purchased them. Comparisons under these conditions show that aging of properly prepared Digitalis Tincture has no apparent effect on the course of the chemical assay, and that high or low results cannot be attributed to this factor.

Daily room temperatures were recorded and these varied from 19.5° to 25.5°. These variations appear to have some effect on the rate of the reaction, but they apparently affect the standard and sample in the same manner and can be discounted.

Five of the eleven Digitalis Tablets yielded tinctures which gave green filtrates in the course of the assay. Four of these tablets were products of the same manufacturer and the green color appeared to have no influence on the course of the reaction. The other product was a green-colored, coated tablet, and its tincture gave an accelerated reaction. This acceleration cannot be attributed to the green color, but rather to the constituents of the coating material.

Discussion of Extraction Studies.—Our fifteen-year-old shaker is very efficient while running, but it demands attention at the most unexpected times. Consequently the agitated extractions were carried out in eight-hour periods. The extractions requiring more than eight hours were interrupted by sixteen-hour periods (overnight) in a refrigerator at 2°. Thus, while with the shortest extraction period the drug was in contact with the menstruum exactly eight hours, with the sixteen-hour and twenty-four-hour preparations the drug was actually in contact with the menstruum thirty-two hours and fifty-six hours, respectively. This arrangement obviously favored the longer extraction periods, but, nevertheless, the eight-hour extraction period in a mechanical shaker appears to have established itself beyond doubt as a period of sufficient length for practically complete removal of the digitalis principles. In order to be doubly certain, the drug and menstruum could be mixed, allowed to macerate overnight, and then be subjected to an eight-hour period in a mechanical shaker.

SUMMARY

1. The Knudson-Dresbach method as modified by Bell and Krantz for the colorimetric determination of digitalis activity has been studied.

2. The use of this method in control laboratories not equipped for biological assay work has been discussed, and is recommended.

3. Extractions of Powdered Digitalis and Digitalis Tablets by the U. S. P. XIII procedure, except for variations in the length of the extraction period, have proved conclusively that under the described conditions an eight-hour extraction in a mechanical shaker will remove practically all of the active digitalis principles soluble in the official menstruum.

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Chemical Differentiation of Desoxyephedrine from Other Sympathomimetic Amines*

By THOMAS J. HALEY†

Methods for the chemical identification and differentiation of isomers of desoxyephedrine from each other and from twelve additional sympathomimetic amines are described. Of the reagents employed, chloroauric, chloroplatinic, and picric acids were most useful since they produced crystalline precipitates having definite melting points.

THE PRESENT commercial availability of potent drugs such as desoxyephedrine and related sympathomimetic amines makes it imperative that an easy method for their identification be made available. Thus far only two cases of toxicity from prolonged use of desoxyephedrine have been reported (2, 4).

Dultz (5) has shown that the drug may be distilled from alkalized cadaveric material and the quantity therein determined by titration. Urinary excretion of desoxyephedrine as well as other similar amines has been determined by Richter (11) with a nonspecific picric acid reagent. Both of the above methods may be used to determine the amount of drug causing death but they will not differentiate between its various forms or between it and other similar amines. Compounds of this type are usually identified by converting them into known derivatives. Of the three forms of desoxyephedrine only the *d*-isomer has been investigated thoroughly in this manner. All reported derivatives of desoxyephedrine are given in Table I. Table II shows the structural differences and the melting points of all the sympathomimetic amine salts studied in this investigation.

This study of the reaction between fifteen sympathomimetic amine salts and ten alkaloidal reagents was undertaken in an attempt to find a rapid method for the identification and differentiation not only of the three isomers of desoxyephedrine but also of the other compounds listed.

TABLE I.—RECORDED DERIVATIVES OF DESOXYEPHEDRINE

Isomer	Derivative	Melting Point, °C.	Reference
Dextro	Hydrochloride	170–171	Ogata (10)
		171–173	Novelli and Tainter (9)
		171–173	Rosenmund, *et al.* (12)
	Bitartrate	118–119	Ogata (10)
	Picrate	144–145	Ogata (10)
	Mercuric Chloride	152–153	Ogata (10)
	Gold Chloride	127	Ogata (10)
	Platinic Chloride	214–215	Ogata (10)
	Picrolonate	183	Dultz (5)
Racemic	Hydrochloride	133–135	Novelli (8)
		132–133	Novelli and Tainter (9)
		134	Rosenmund, *et al.* (12)
	Platinic Chloride	198–199	Erlenmeyer and Simon (6)
	Thiourea	134	Erlenmeyer and Simon (6)
	Sulfathiazole	118–120	Hamilton, *et al.* (7)
	Sulfadiazine	187–189	Hamilton, *et al.* (7)
Levo	Tartrate	159–160	Novelli and Tainter (9)

EXPERIMENTAL

All the compounds tested were made up in one per cent solutions; the alkaloidal reagents were prepared as directed by Autenrieth and Bauer (1), and the reagents for the Copper Sulfate test according to the directions in the United States Pharmacopœia (13). One cubic centimeter of the amine solution to be tested was placed in a small test tube and four

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TABLE II.—STRUCTURAL DIFFERENCES OF THE AMINES TESTED

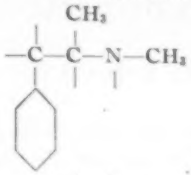
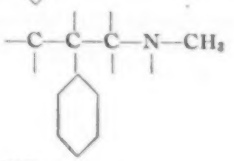
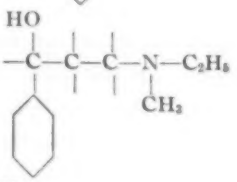
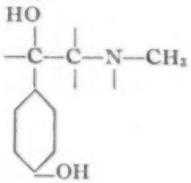
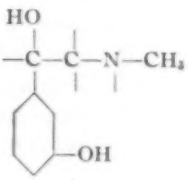
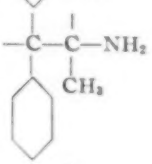
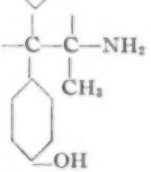
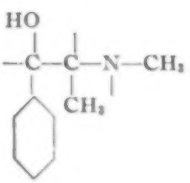
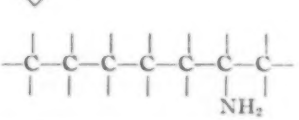
Amine	Structural Formula	Melting Point of Salts
Desoxyephedrine (1-phenyl-2-methylaminopropane)		*d*-Hydrochloride 170–173° *dl*-Hydrochloride 132–135° *l*-Tartrate 159–160°
Vonedrine (2-phenyl-1-methylaminopropane)		*dl*-Hydrochloride 144–148°
Nethamine (1-phenyl-3-methylethylamino-1-propanol)		*dl*-Hydrochloride 186°
Synephrine (Sympatol) (1-(*p*-hydroxyphenyl)-2-methylaminoethanol)		*dl*-Tartrate 183–185°
Neo-Synephrine (1-(*m*-hydroxyphenyl)-2-methylaminoethanol)		*d*-Tartrate 98–100° *dl*-Hydrochloride 142–143° *l*-Hydrochloride 141.5–143°
Benzedrine (1-phenyl-2-aminopropane)		*dl*-Sulfate above 300° *d*-Sulfate above 300° (Dexedrine)
Paredrine (1-(*p*-hydroxyphenyl)-2-aminopropane)		Hydrobromide 189–190°
Ephedrine (1-phenyl-2-methylamino-1-propanol)		*dl*-Hydrochloride 187–188° *l*-Sulfate 240–247.5°
Tuamine (2-aminoheptane)		Sulfate 275–277°

TABLE III—REACTIONS BETWEEN ALKALOIDAL REAGENTS AND SYMPATHOMIMETIC AMINES

Amine	U. S. P. $CuSO_4$ Test	Chloro-auric Acid, 1:20	Chloro-platinic Acid, 5%	Silico-tungstic Acid, 5%	Phospho-molybdic Acid, 10%	Phospho-tungstic Acid	Mayer's Reagent	Mercuric Chloride, 1:20	Iodo-Potassium Iodide Solution (Wagner's Reagent)	Saturated Aqueous Solution of Picric Acid
-Ephedrine Sulfate	Purple ether layer	Negative	Negative	Amorphous white precipitate	Amorphous yellow precipitate	Negative	Slight precipitate on standing	Negative	Amorphous brown precipitate	Negative
dl-Ephedrine Hydrochloride	Purple ether layer	Negative	Negative	Amorphous white precipitate	Amorphous yellow precipitate	Negative	Negative	Negative	Negative	Negative
dl-Desoxyephedrine Hydrochloride	Negative	Yellow needles 115°	Orange needles 199°	Amorphous white precipitate	Amorphous yellow precipitate	Amorphous white precipitate 186–195°	Amorphous yellow precipitate	Colorless crystals on standing 126°	Amorphous black precipitate	Yellow needles 118°
d-Desoxyephedrine Hydrochloride	Negative	Yellow needles 130°	Orange needles 212°	Amorphous white precipitate	Amorphous yellow precipitate	Amorphous white precipitate 206–216° d.	Amorphous yellow precipitate	Colorless crystals on standing 156°	Amorphous brown precipitate	Yellow needles 146°
-Desoxyephedrine Tartrate	Negative	Yellow needles 126°	Orange needles 215° d.	Amorphous white precipitate	Amorphous yellow precipitate	Amorphous white precipitate	Amorphous yellow precipitate	Negative	Amorphous brown precipitate	Yellow needles 144°
Vonedrine Hydrochloride	Negative precipitate reduced	Yellow needles 158°	Orange needles on standing 200°	Amorphous white precipitate	Amorphous yellow precipitate	Negative	Amorphous yellow precipitate	Colorless crystals on standing 168°	Amorphous brown precipitate	Yellow needles on standing 153°
Nethamine Hydrochloride	Negative	Yellow needles on standing 109°	Negative	Amorphous white precipitate 145–150° d.	Amorphous yellow precipitate	Negative	Amorphous yellow precipitate	Negative	Amorphous reddish-brown precipitate	Amorphous yellow precipitate
dl-Benzedrine Sulfate	Negative	Negative	Negative	Amorphous white precipitate 209–220° d.	Amorphous yellow precipitate	Amorphous white precipitate	Negative	Negative	Negative	Negative
d-Benzedrine (Dexedrine Sulfate	Negative	Negative	Negative	Amorphous white precipitate 210–216° d.	Amorphous yellow precipitate	Amorphous white precipitate	Negative	Negative	Negative	Negative
Paredrine Hydrobromide	Negative	Solution turned blood-red	Negative	Negative	Brown crystals on standing 190–210° d.	Negative	Negative	Negative	Negative	Negative
Synephrine Tartrate	Negative	Negative	Negative	Negative	Amorphous yellow precipitate immediately soluble	Negative	Negative	Negative	Negative	Negative
dl-Neo-Synephrine Hydrochloride	Purple Ether layer	Negative	Negative	Negative	Amorphous brown-yellow precipitate	Negative	Negative	Negative	Negative	Negative
l-Neo-Synephrine Hydrochloride	Purple Ether layer	Negative	Negative	Negative	Amorphous brown-yellow precipitate	Negative	Negative	Negative	Negative	Negative
d-Neo-Synephrine Tartrate	Negative	Negative	Negative	Negative	Amorphous yellow precipitate immediately soluble	Negative	Negative	Negative	Negative	Negative
Tuamine Sulfate	Negative precipitate reduced	Yellow needles on standing 80°	Negative	Amorphous white precipitate	Amorphous yellow precipitate	Amorphous white precipitate 225–250°.	Negative	Negative	Negative	Yellow needles 106°

drops of the reagent added. Observations were made immediately and after one, two, three, four, and twenty-four hours. The results with all the amines tested are given in Table III. Where crystalline or amorphous precipitates were formed, they were filtered, placed in a vacuum desiccator over P_2O_5 two hours, and their melting points then determined using a Thiele tube. All melting points reported are uncorrected.

DISCUSSION

Chen (3) investigating the $CuSO_4$-NaOH test on a series of twenty-seven aromatic amines, including six optical isomers of ephedrine, found that those having an OH group on the second C atom from the amine group gave a positive test. By comparison of Tables II and III it may be seen that both the *l*- and *dl*-ephedrines and *l*- and *dl*-Neosynephrines gave a positive purple coloration which, in the case of the latter, was not ether soluble. The negative test given by *d*-Neosynephrine, as well as Synephrine, may have been due to the use of their tartaric acid salts. This acid probably interferes by preferentially reacting with the alkaline copper sulfate as it does in Fehling's solution. If inorganic acid salts were employed, both *d*-Neosynephrine and Synephrine would give a positive test. The results obtained with the other amines were to be expected because they did not have the desired group.

The results obtained with phosphomolybdic and silicotungstic acids were similar to those described for other compounds by Autenrieth and Bauer (1). In those instances where precipitates were formed, they were generally so unstable that they could not be isolated before decomposition. Also, the fact that all the precipitates were the same color prevents the use of this test for identification purposes. The phosphotungstates isolated had no definite melting points and thus were of little value in identifying the amines.

All isomers of desoxyephedrine, as well as both Vonedrine and Nethamine, reacted with most of the reagents employed, and it was only by the use of melting point differences that these drugs could be identified. The crystalline precipitates formed with chloroauric and chloroplatinic acids appeared immediately, could be filtered and dried rapidly and form the basis of a rapid method of identification and differentiation. The other crystalline precipitates which formed upon standing would add further evidence as to the exact amine causing the toxic symptoms or death.

Where "Negative" appears in Table III, it should not be concluded that no reaction occurred with the reagent, since the derivatives may have been formed but were too soluble to precipitate out of solution. The failure of *l*-desoxyephedrine to give a precipitate with mercuric chloride must be ascribed to persistent supersaturation of the system in the absence of the solid phase rather than to the greater solubility of the "*l*"-isomer derivative.

SUMMARY

1. A method has been described for the chemical identification and differentiation of the isomers of desoxyephedrine from each other and from twelve other sympathomimetic amines.

2. Four new derivatives of *dl*-desoxyephedrine and three new derivatives of *l*-desoxyephedrine have been described.

3. Chloroauric, chloroplatinic, and picric acids were the most useful of the reagents employed with the sympathomimetic amines because they gave crystalline precipitates having definite melting points. Further, they form these precipitates quickly and thus enable a rapid identification to be made.

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A Graphical Calculator for Bioassays*

By FRANK M. GOYAN and J. DUFRENOY †

A new graphical calculator built as a two-dimensional slide rule has been designed to expedite the plotting and reading of reference curves for bioassays. This device has been applied to the assay of penicillin using logarithmic scales for both the concentrations of pencillin and the diameters of the zones of inhibition.

PENICILLIN ASSAY by the standard cup-plate method is accomplished by establishing the relationship between zone size (in mm.) and concentration of penicillin in Oxford units per cc. (u./cc.). Several investigators have described graphical methods of computing concentration from zone size based upon simple arithmetic functions (1–3). So-called penicillin slide rules now in common use are based upon this principle (4).

In an attempt to obtain a linear function E. J. de Beer, M. B. Sherwood (5), L. F. Knudsen, W. A. Randall (6), and C. I. Bliss (7) substituted log concentration for concentration. Knudsen and Randall stressed that "It has been shown repeatedly at several laboratories that, for doses of penicillin equivalent to from 0.25 unit per ml. to 3 units per ml. in each cup, there is a straight line relationship between the diameter of the zone of inhibition and the logarithm of the dose. In other words, if the log of the dose is plotted against the diameter of the zone of inhibition, the points indicate a straight line." Assuming this straight line relationship to be valid, determination of potencies depends mainly on the determination of the slope of the line for two known dilutions of the standard and two corresponding dilutions of the unknown. To that end Knudsen and Randall (6) published a chart for determining potency as per cent of standard from the two-concentration plate method and Bliss (7) published charts for penicillin assay based on two test solutions for both the standard and the unknown. One of us in connection with the study of a great number of routine penicillin assays, found it advantageous to plot log concentrations against log diameters: the "standard" curve is then represented by two straight lines, one sloping more steeply in the range from 0.5 u./cc. to 1.5 u./cc., and the other sloping less steeply from 1.5 u./cc. to 4 u./cc.[1] Excellent agreement was obtained between concentrations read by standard methods (using a reference curve plotted on arithmetic paper) and those read from the logarithmic plot.

Reading the zones from plates submitted to the "physical development" previously described (8) made it possible to graph the "log concentration-log diameter" reference curve as a straight line throughout the range from 1 to 8 u./cc. However, the use of commercially available logarithmic paper entails various difficulties. To remove these difficulties one of us devised a two-dimensional slide rule which performs the mathematical function of a special logarithmic paper, with the additional advantage (among others) that the dilution factors can be read directly (see scale *B* of Fig. 1) and the standard zone sizes set up without redrawing the curve, simply by moving the panel *P* up or down in relation to the ordinate scale *O*.

DESCRIPTION OF APPARATUS

The graphical calculator or two-dimensional slide rule shown in Fig. 1 was constructed as follows:

A 14″ × 12″ piece of plywood (*F*) is used as the support of the frame; the basal part of the frame consists of a slide rule, made by screwing on the 14″ × 12″ piece of plywood two pieces of wood (*G*) which are beveled so as to form the groove in which the beveled ruler (*B*) slides freely; the sides of the frame (*F*) are made up each of a piece of beveled plywood, allowing a beveled panel (*P*) (also of plywood) to slide up and down.

The ruler (*O*), bearing the ordinate scale, is secured to the ruler (*B*) by means of a T-shaped piece of brass which is built up to serve as a handle for

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[1] For instance, in actual tests the slope was calculated as (log 23.5 − log 18.09)/(log 1.5 − log 0.5) = 0.24 for the steeper straight line in the range from 0.5 to 1.5 u./cc. and as (log 27.5 − log 23.5)/(log 4 − log 1.5) = 0.15 for the less steep straight line in the range from 1.5 to 4 u./cc.

sliding ruler (*B*) and attached ordinate scale. To the basal part of the frame is then glued a strip cut from two-cycle log paper. This strip is wide enough to carry two sets of graduations of the *A* scale. The upper divisions are lettered in black from 0.1 to 10 u./cc.; directly below, the same divisions are lettered in red from 1 to 100 u./cc. Below these two logarithmic scales there might be added a corresponding linear scale to be used in other types of calculations.

native scales may be secured on the vertical ruler (*O*) in such manner that any one can be selected by folding top ones back, like opening a book. A linear vertical scale may be used with equal success and would be in keeping with the recommendations of several authorities (5–7) who have shown that log dose plotted against diameter of inhibition zone will give a linear relationship for results obtained with the standard cup-plate assay. However, this instrument was developed for the new three-hour assay

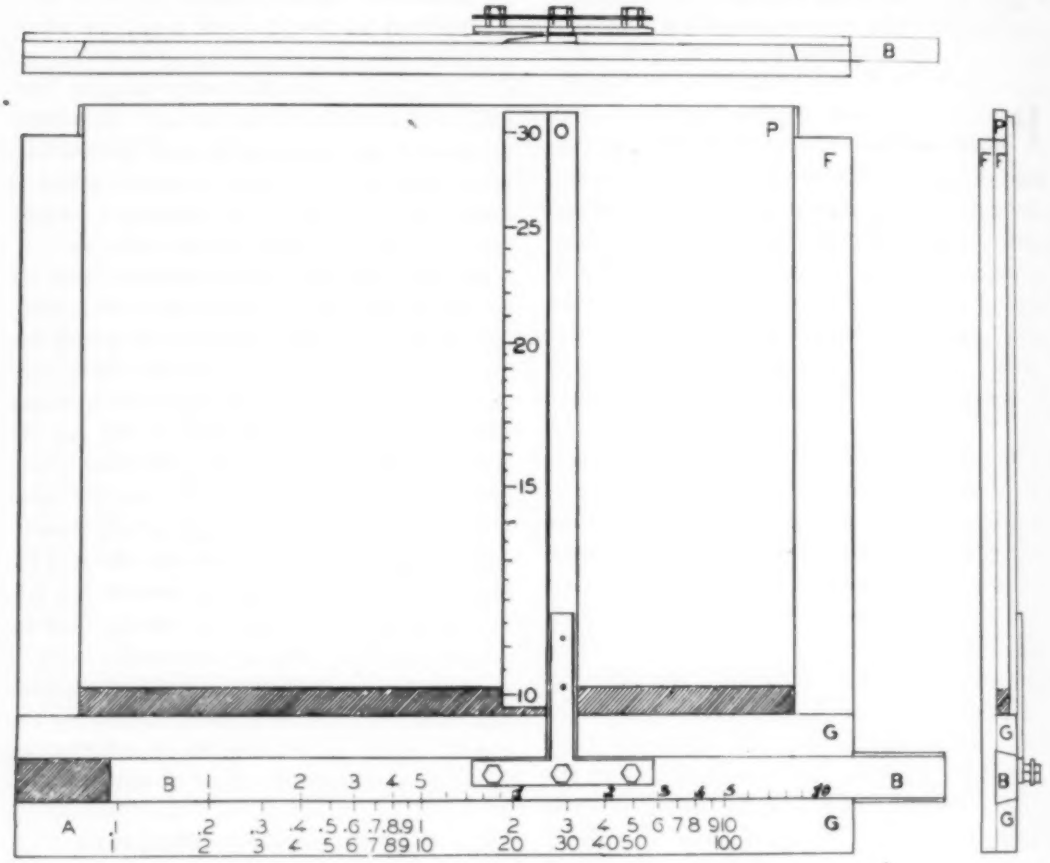

Fig. 1.—Detail drawing of graphical calculator.

A matching strip of the two-cycle log paper is glued to the movable ruler (*B*). The first cycle should be lettered from 1 to 10 in red and the second cycle from 1 to 10 in black so that the operator may match color between scales *A* and *B* and thus avoid confusion in placing the decimal point. A cursor may be fastened to the brass connector so that the reading line falls at the beginning of the second cycle on scale *B*.

To the vertical ruler is affixed a segment of a one-cycle log scale so chosen that the slope of the calibration curve plotted on board (*P*) shall fall at an angle of approximately 45°. When using cups 8 mm. in external diameter the diameters of zones of inhibitions range from 10 mm. to 30 mm.; therefore, for this type of cup-plate assay the usable part of the scale ranges from 10 to 30 at most. However, alter-

(8) and was found to work equally well with data obtained from the standard assay. The operation of the graphical calculator is not dependent upon strict linearity of the reference curve nor upon maintaining a log-log relationship between the vertical and horizontal scales.

The drawing board (*P*), to which unruled paper is fastened with adhesive cellulose tape, must be adjusted so that it can slide up and down over a sufficient range that, once a standard curve is established, it may be shifted to accommodate daily variations in the size of the standard zone.[2]

[2] The curve or line may also be shifted by slightly rotating the paper about any axis when it is desired to make use of the "two standard method." No mechanical aid has been provided in the present model to accomplish this, although a pin may be used to establish any desired axis of rotation.

The use of the calculator involves two steps: first, the plotting of a standard curve; second, reading concentrations of solutions under test from measured zone diameters. Once the reference curve is established it becomes an integral part of the calculator and may be used from day to day by sliding the panel *P* to the position indicated for the day by the size of the standard zone.

To Plot a Reference Curve.—Set the origin (1 at beginning of second cycle) of movable scale *B*, to face any known value of u./cc. on scale *A*. Record corresponding observed values of diameters of zones of inhibition on ordinate scale *O*, by marking a point on unruled paper attached to drawing board *P*.[3] Repeat for each known value. Two examples of the plotting of typical reference curves are given below.

Example 1.—Using data from Table I plot the reference curve.

TABLE I.—AVERAGE RESULTS FROM CUP-PLATE ASSAYS[a]

Known Concentrations of Penicillin, U./Cc.	Measured Zones Diameters, Mm.
0.25	14.80 ± 0.16
0.50	18.47 ± 0.14
0.75	20.43 ± 0.16
1.00	21.86 ± 0.13
1.50	23.59 ± 0.16
2.00	24.90 ± 0.16
3.00	26.50 ± 0.17
4.00	27.55 ± 0.19

[a] Routine measurements made at Cutter Laboratories by the standard Oxford cup-plate method according to the specifications of the Food and Drug Administration, using *Staph. aureus* F.D.A. strain No. 209P as the test organism. Each zone diameter represents an average taken from fifty daily standard curves.

[3] The frame of the calculator is assembled with machine screws and can be adjusted so that board (*P*) has no tendency to slip accidentally while plotting a curve.

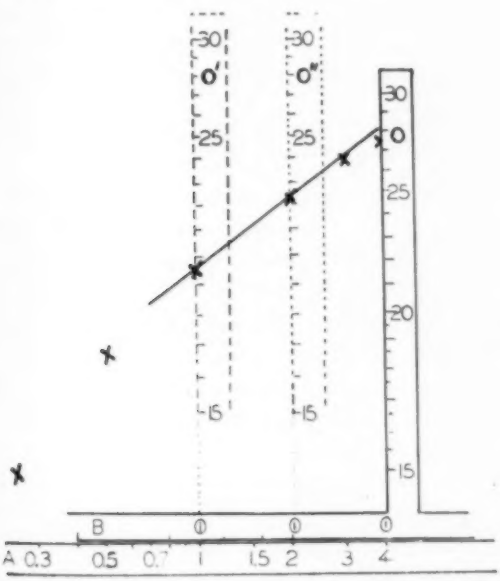

Fig. 2.—Reference curve for standard penicillin assay. The dotted scales O′ and O″ are set, respectively, for adjusting the instrument to a new standard and for reading an unknown concentration. (See Table I and Examples 1 and 3 in text.)

Secure paper to drawing board (*P*) which should be placed as shown in Fig. 1. Move the origin of scale *B* (beginning of second cycle of scale *B* shown in Fig. 1) successively to the values given in the first column of Table I. For each position a pencil mark is placed directly under the corresponding zone diameter, estimated on scale *O*.

Figure 2 shows this curve as plotted on paper attached to board *P;* the scale *O*, at the extreme right, shows the position of this scale and the attached scale *B* after plotting data of Table I.

Example 2.—Using the data from Table II plot the reference curve.

TABLE II.—AVERAGE RESULTS FROM 118 PHYSICALLY DEVELOPED PENICILLIN ASSAY PLATES

Penicillin, U./Cc.	Observed	Diameters of Zones of Inhibition Calculated[a]
0.50	13.28 ± 0.08	...
1.00	14.91 ± 0.08	14.93
2.00	16.23 ± 0.08	16.16
4.00	17.30 ± 0.08	17.45
8.00	18.93 ± 0.10	18.87

[a] Using first-degree orthogonal polynomials, as from Fisher and Yates, "Statistical Tables for Biological, Agricultural and Medical Research," through Anderson and Houseman (9).

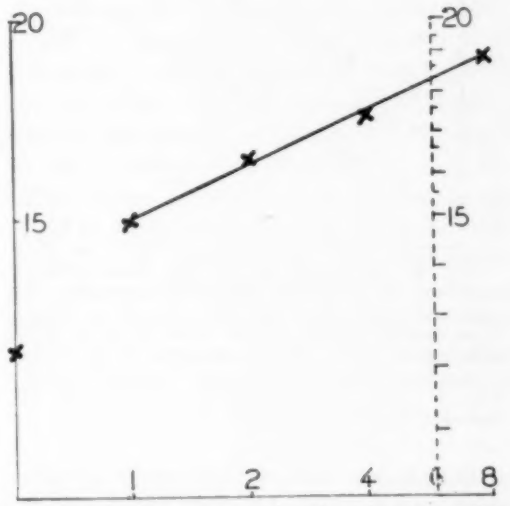

Fig. 3.—Reference curve for three-hour penicillin assay. The dotted scale is set for the reading of an unknown solution producing a zone 18.26 mm. in diameter. The concentration is seen to be 6 u./cc. Refer to Table II and Examples 2 and 4 of text.

This curve is plotted by following the procedure outlined in Example 2. Figure 3 shows the completed curve.

To Determine the Potency of Solution under Test.—(*a*) When the proper reference curve is on board *P*, move the scale *B* until the origin appears directly opposite the reading on the *A* scale equal to the value of the concentration of the standard solution of the day. (*b*) Slide the drawing board *P* up or down to set the reference curve directly under the value of the diameter of the standard

zone as read on the ordinate scale *O*. (*c*) Slide movable ruler *B* left or right until the reference curve appears directly under the division of the scale *O* corresponding to the measured diameter of the inhibition zone for the dilution under test. Then read u./cc. on scale *A* opposite the proper dilution factor on scale *B*. It will be noted that the use of dilution factors on scale *B* corresponds to simple multiplication with the *A* and *B* scales of ordinary slide rules.

Example 3.—Using the reference curve described in Example 1 and shown in Fig. 2 compute the concentration of penicillin corresponding to a zone size of 22.25 mm. when the average zone diameter for cups containing 1 u./cc. on the same set of plates is 19.6 mm.

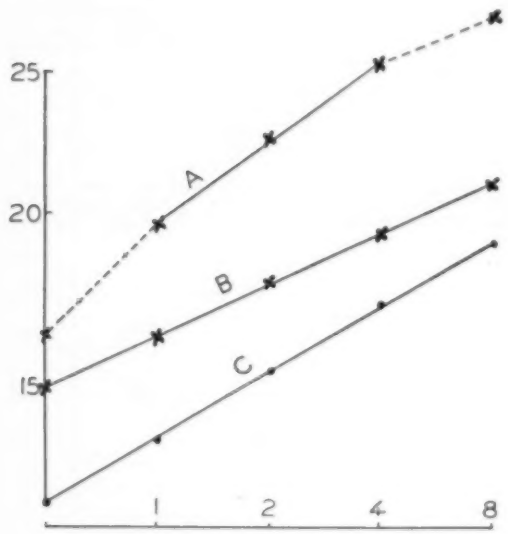

Fig. 4.—Reference curves for three different methods of cup-plate assay for penicillin. *A*, standard sixteen-hour method; *B* and *C*, physical development method (8); *B*, three hours' penicillin diffusion on plates incubated with *Staph. aureus*; *C*, two hours' penicillin diffusion on plates incubated with *B. subtilis*. The scales are the same as those shown in Fig. 1.

To make use of the unit zone given in Example 3 move scale *B* so that the origin corresponds with 1 u./cc. on scale *A*, then move board *P* so that the reference curve appears under 19.6 (position *O'*, Fig. 2). Without further movement of board *P* slide scale *B* until 22.25 as estimated on scale *O* coincides with the line; read the required concentration of 2 u./cc. opposite the origin of scale *B* (position *O"*, Fig. 2).

Example 4.—Using the reference curve mentioned in Example 2 and illustrated in Fig. 3 determine the concentration corresponding to a zone diameter of 18.26 mm. if a concentration of 1 u./cc. produces a zone of 14.9 mm. in diameter.

Figure 3 shows the calibration curve plotted as described above, the unit concentration is set at 14.93 which is practically equivalent to 14.9 so that it is not necessary to move board *P* to reset the standard, thus the concentration at 6.00 u./cc. is read directly by bringing scale *O* to the position shown in Fig. 3. If it is known, for example, that this solution was obtained by dilution, the value of the undiluted solution would be found opposite the known dilution factor.

ADVANTAGES OF THE GRAPHICAL CALCULATOR

The graphical calculator described above has been in regular use in our laboratory since July, 1946, and has proved satisfactory for different types of assays. It has been used effectively even when the function to be treated was not linear. By selecting the proper ordinate scale and by placing it in such a position that it is possible to cover any desired range, a variety of functions may be set up for the graphical computation of assays. Figure 4 shows three examples of different calibration curves which can be used on the same sheet of paper. This device also serves nicely as a means of plotting and recording results for comparison and reference.

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A Graphical Calculator for Statistical Analysis*

By J. DUFRENOY and FRANK M. GOYAN†

The graphical calculator originally developed for calculating penicillin concentrations from zone diameters in the three-hour cylinder-plate assay is shown to possess properties that recommend it for statistical analysis. By the introduction of two additional scales, the instrument is fitted to test data for deviation from normal distribution and to determine the mean and standard deviation from the mean of normally distributed values. Examples are included to demonstrate the advantages of graphical statistical analysis. A device for the semiautomatic plotting of data may be added if desired. The instrument is also useful in calculating values of LD_{50} from suitable data.

THE GRAPHICAL calculator previously described as expediting the plotting of reference curves for bioassays (1) can be adapted to the plotting of various functions by introducing the proper graduations on the horizontal and vertical scales. For the purpose of elementary statistical analysis, a probability scale, which may be made from a strip cut from a sheet of commercially available probability paper (2–4), is added as an additional horizontal scale, and a linear scale is provided for the vertical ruler. Thus equipped, the graphical calculator permits a direct visual test for deviations from normal distribution, together with direct reading of the mean and a rapid estimate of standard deviation from the mean of data that may be assumed to be normally distributed. The standard error of the mean or average ($S.E._{av.}$) is obtained from the standard deviation (σ) by the use of the equation,

$$(S.E._{av.}) = \sigma/\sqrt{n} \qquad (I)$$

where n represents the total number of quantities included in the average.

The theoretical justification and the significance of this method of calculating statistics are based upon well-established principles. The graphical calculator, modified as described above, exhibits the usual mathematical properties of arithmetic probability graph paper, which is commonly used in testing for normal distribution (3–5). Direct reading of mean values is possible because of the relationship between the mode of the normal distribution curve and the mid-point of the probability scale. Values of the mean *minus* the standard deviation, and values of the mean *plus* the standard deviation, are read directly opposite two fixed points on the probability scale. The position of these two points lying near the 16 per cent and the 84 per cent positions, respectively, is established from the fact that 68.27 per cent of normally distributed values fall symmetrically about the mean within the range established by values either greater or less than the mean by an amount equal to the standard deviation. Values determined in this way for the standard deviation are converted to the standard error of the average in the usual way; for example, as defined in the U. S. P. XIII monograph on Digitalis Tincture (6). It can be shown that this definition is equivalent to Eq. (I) by assuming that values of the standard deviation as read from the calculator are equivalent to values for the standard error, or the square root of the variance. The terms *normal distribution*, *standard error*, *standard deviation*, and *variance* are used in the sense established by modern treatises on statistical methods (7–9).

Although the graphical calculator offers no theoretical advantage over a sheet of probability paper, it provides a definite practical advantage in addition to the obvious one of greater convenience in plotting. This advantage arises from the fact that it is possible to designate fixed positions on the probability scale for various total numbers of values to be included in an average. These positions may be marked on the scale, or a mechanism consisting of a spring-activated plunger operating against a metal

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guide may be attached to the instrument in such a way that any selected series of positions on the probability scale is located automatically in the course of plotting the data. For relatively small populations, the only preliminary operation necessary to the operation of the instrument is to arrange the set of values to be studied in serial order, the smallest first, etc. Larger populations require preliminary grouping unless it is deemed safe to consider portions of the populations of values separately.

CONSTRUCTION OF THE GRAPHICAL CALCULATOR

The graphical calculator previously described for calculating the results of bioassays (1) is very easily modified for elementary statistical analysis. The essential new features shown in Fig. 1 are the horizontal probability scale (*A*), the vertical linear scale (*O*), and the cursor (*C*). The log scales described previously as occupying positions *A* and *B* are omitted from the drawing for the sake of clarity of presentation, although it is not desirable to remove these scales when modifying the instrument. Instead, the instrument is made to serve a double purpose by cementing a strip of probability paper below the log scales. The cursor (*C*) is provided for accurately reading the probability scale.

The linear vertical scale is made by fastening a strip of millimeter paper, or any other suitable linear scale, to the vertical ruler (*O*). It is necessary to provide for the temporary marking of the numerical values chosen for the graduations on this scale because each new problem may require a different range of values. The simplest method, of course, is to write numbers on the scale in pencil and to erase them when necessary. This scale should be constructed in a form that will permit it to be interchangeable with one or more log scales, which may prove useful for a wide variety of calculations.

Marking the Probability Scale.—In addition to the usual percentage values found on all probability scales, it is desirable to provide permanent or semipermanent marks on the probability scale itself. These marks indicate the position of the cursor (*C*) when plotting data or reading the mean, the mean minus the standard deviation, or the mean plus the standard deviation. Such marks are made at the 50%, the 15.86%, and the 84.14% positions, respectively, to indicate the three reading positions.

The positions to be marked for plotting data may be calculated for each number of values likely to be encountered. Table I gives the location of these plotting points for odd total numbers of data from 3 to 25, inclusive. Marked points for even numbers of values are not required because no serious error is introduced by skipping the mid-point while plotting even numbers of data by making use of the positions established for the next higher odd number. Calculations of the entries in Table I are based upon the

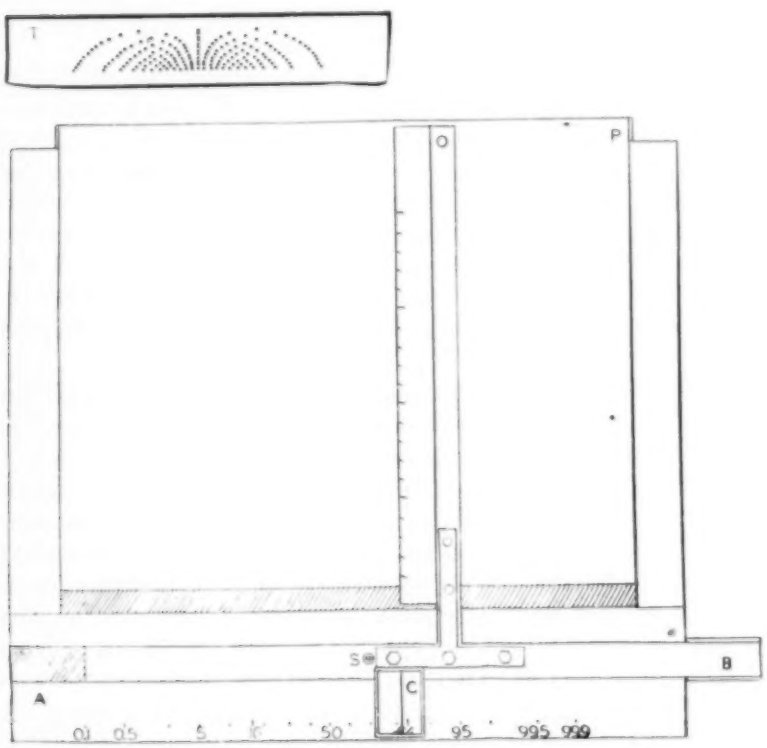

Fig. 1.—Top view of the graphical calculator adapted for the determination of the mean and standard deviation of a series of measurements assumed to be normally distributed. The insert T (not drawn to scale) shows the pattern of holes in a metal guide used to provide semi-automatic plotting.

idea of selecting the mid-point of the cumulative percentage range corresponding to each one of the population of values under consideration. For example, out of a population of five values, the first value represents 20%, and the mid-point of the range is 10%; the next value is thought to occupy the range from 20% to 40%, the mid-interval being the 30% position; the next position is 50%, etc. Twelve separate lines below the main probability scale would be required for marking the positions indicated by the columns of Table I, but if a color code is employed, two lines can be consolidated with some sacrifice of clarity. Each line must be labeled to show what points are to be placed under the reading line of cursor *C* when plotting a set of data containing the number of values indicated by the label.

TABLE I.—LOCATION OF SUCCESSIVE POSITIONS ON THE PROBABILITY SCALE FOR PLOTTING ODD NUMBERS OF DATA IN GROUPS OF 3 TO 25

Serial No.	Number of Data											
	3	5	7	9	11	13	15	17	19	21	23	25
1	16.7	10.0	7.1	5.6	4.5	3.8	3.3	2.9	2.6	2.4	2.2	2.0
2	50.0	30.0	21.4	16.7	13.6	11.5	10.0	8.8	7.9	7.1	6.5	6.0
3	83.3	50.0	35.7	27.8	22.7	19.2	16.7	14.7	13.1	11.9	10.9	10.0
4	...	70.0	50.0	38.9	31.8	26.9	23.3	20.6	18.4	16.7	15.2	14.0
5	...	90.0	64.3	50.0	40.9	34.6	30.0	26.5	23.7	21.4	19.6	18.0
6	...	...	78.6	61.1	50.0	42.3	36.7	32.3	28.9	26.2	23.9	22.0
7	...	...	92.9	72.2	59.1	50.0	43.3	38.2	34.2	31.0	28.3	26.0
8	...	...	...	83.3	68.2	57.7	50.0	44.1	39.5	35.7	32.6	30.0
9	...	...	...	94.4	77.3	65.4	56.7	50.0	44.8	40.5	37.0	34.0
10	...	...	...	...	86.4	73.1	63.3	55.9	50.0	45.2	41.3	38.0
11	...	...	...	...	95.5	80.8	70.0	61.8	55.2	50.0	45.6	42.0
12	...	...	...	...	...	88.5	76.7	67.7	60.5	54.8	50.0	46.0
13	...	...	...	...	...	96.2	83.3	73.5	65.8	59.5	54.4	50.0
14	...	...	...	...	...	...	90.0	79.4	71.1	64.3	58.7	54.0
15	...	...	...	...	...	...	96.7	85.3	76.3	69.0	63.0	58.0
16	...	...	...	...	...	...	...	91.2	81.6	73.8	67.4	62.0
17	...	...	...	...	...	...	...	97.1	86.9	78.6	71.7	66.0
18	...	...	...	...	...	...	...	...	92.1	83.3	76.1	70.0
19	...	...	...	...	...	...	...	...	97.4	88.1	80.4	74.0
20	...	...	...	...	...	...	...	...	...	92.8	84.8	78.0
21	...	...	...	...	...	...	...	...	...	97.6	89.1	82.0
22	...	...	...	...	...	...	...	...	...	...	93.5	86.0
23	...	...	...	...	...	...	...	...	...	...	97.8	90.0
24	...	...	...	...	...	...	...	...	...	...	...	94.0
25	...	...	...	...	...	...	...	...	...	...	...	98.0

Mechanism for Automatic Plotting.—Table I was used in preparing the metal guide shown in the insert (*T*) of Fig. 1. The pattern of dots represents holes drilled in a piece of sheet brass at positions determined by plotting entries in Table I on probability paper cemented to the brass; each column was plotted on a separate horizontal line. By means of springs, the guide is held in contact with the under surface of the calculator directly under the plunger (*S*) in such a way that its longer dimension is parallel to the probability scale. It may be moved forward or backward in a direction perpendicular to the scale. An extension of this metal guide (not shown) is provided with holes that engage pins secured to the base of the calculator. This precaution eliminates the possibility of a shift in the position of the guide once it has been properly placed as determined by an auxiliary scale attached to the extension.

The plunger (*S*) is constructed from a short length of steel rod chosen so that it slides freely up and down through a piece of brass tubing which is forced through a perpendicular hole in the moving part (*B*). A slot is cut under the path of the plunger through the base of the calculator; horizontal movement of the sliding ruler (*B*) causes the rounded tip of the plunger to slide from one hole in the guide to the next, coming to rest in each successive hole of whatever horizontal line of the guide was selected. The tension of the coil-spring holding the plunger against the guide is so adjusted that the sliding ruler (*B*) comes to a stop each time the rounded end of the plunger comes to rest in one of the holes in the metal guide; slight additional pressure, tending to slide the ruler (*B*), causes the plunger to progress horizontally to the next position.

USE OF THE GRAPHICAL CALCULATOR

Treatment of Data Without the Use of Table I.—The first step in using the graphical calculator is to arrange all of the values to be studied as a single group, in serial order of increasing or decreasing magnitude. It is a well-known property of the normal distribution that, out of one hundred values serially arranged, the first and last sixteen will normally have values that deviate from the mean by amounts equal to or greater than the standard deviation. As an example, measured diameters of one hundred inhibition zones produced by the action of penicillin solutions of unit concentration in the standard cylinder-plate assay are serially arranged in Table II. The fiftieth value is 21.50 which corresponds to the value of the arithmetic mean (21.51). The sixteenth value is 20.75 which agrees with the value 20.76 calculated by subtracting the value of the

standard error (computed by conventional methods) from the mean. The eighty-fifth value is 22.25 which is in agreement with the value calculated by adding the standard error to the mean.

The selection of one hundred measurements was made to illustrate some of the properties of the probability scale. For one hundred measurements, each measurement accounts for 1% on the probability scale, although the actual point is placed at mid-intervals. For example, the first point is plotted at a position corresponding to the 0.5% division on the probability scale. The second point is plotted to correspond to the 1.5% position; the fiftieth point, to the 49.5% position; etc. This principle may be applied to any number of points after computing, from the serial number of each point, the corresponding percentage value. The method of making this calculation is discussed in connection with Table I.

TABLE II.—DIAMETER (x) OF 100 INHIBITION ZONES PRODUCED BY PENICILLIN SOLUTIONS OF UNIT CONCENTRATION IN THE STANDARD CYLINDER-PLATE ASSAY[a]

x Mm.	x Mm.	x Mm.	x Mm.
19.70	21.10	21.50	21.90
19.80	21.15	21.50	21.95
19.90	21.15	21.55	21.95
20.10	21.15	21.55	21.95
20.20	21.20	21.55	22.00
20.30	21.20	21.55	22.00
20.40	21.25	21.60	22.00
20.50	21.25	21.60	22.10
20.55	21.30	21.60	22.20[d]
20.55	21.30	21.65	22.25
20.60	21.30	21.65	22.25
20.65	21.35	21.70	22.30
20.65	21.35	21.70	22.35
20.70	21.35	21.70	22.40
20.75	21.35	21.75	22.45
20.75[b]	21.40	21.75	22.50
20.80	21.40	21.75	22.55
20.85	21.45	21.80	22.55
20.85	21.45	21.80	22.70
20.85	21.45	21.80	22.80
20.85	21.45	21.85	22.90
20.90	21.50	21.85	22.95
20.95	21.50	21.85	23.20
21.10	21.50	21.90	23.30
21.10	21.50[c]	21.90	23.40

[a] Random selection from routine assays serially arranged. Data from 4 consecutive months, Cutter Laboratories.
[b] 16th value. [c] 50th value. [d] 84th value.

The first step in plotting the points corresponding to the zone diameters listed in Table II is to mark the linear vertical scale (O) in the manner indicated by the ordinate scale shown in Fig. 2, and to secure a piece of paper to the drawing board of the instrument. The vertical scale moves with the cursor (C) which is placed so its reading line coincides with the 0.5% position on the probability scale; the first point is plotted opposite a division of the vertical scale corresponding to a zone diameter of 19.70 mm. Each successive point is plotted from Table II in the same manner at intervals of 1% on the probability scale;

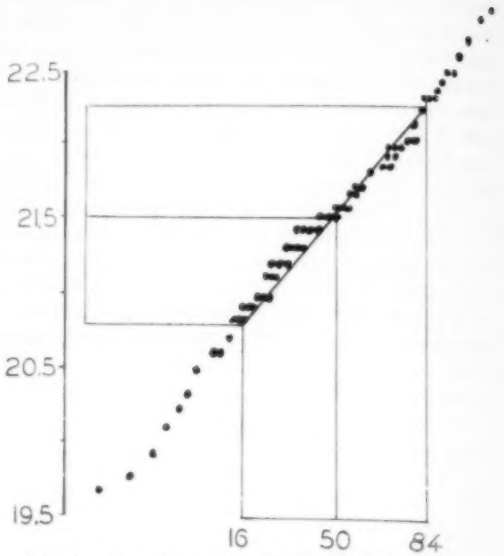

Fig. 2.—Graphical determination of the mean and standard deviation of the diameter of 100 inhibition zones from the standard cylinder-plate assay of penicillin solutions containing 1 unit/cc. The abscissa represents the probability scale; the ordinate, zone diameters (mm.).

the second point, at 1.5%; the third, at 2.5%; the next, at 3.5%; etc., up to 99.5% on the probability scale.

The best fitting straight line is drawn through the points as shown in Fig. 2. The cursor is set opposite the 50% division on the probability scale for the purpose of reading the mean value at the intersection of this line with the vertical scale. From Fig. 2 it may be observed that the value of the mean is read at 21.5 mm. The cursor is then set above the reading mark near the 16% graduation of the probability scale, and the corresponding value of the mean minus the standard deviation is read on the linear scale as 20.8 mm. Therefore, the value of the standard deviation from the mean is 21.5 − 20.8, or 0.7 mm. A similar reading is made when the cursor coincides with the reading mark near the 84% division of the probability scale, and the value of 22.2 mm. is noted. This checks the previous reading, and it will be observed that the standard deviation from the mean is one-half of the difference between the 84% reading (22.2) and the 16% reading (20.8).

Treatment of Data with the Aids Derived from Table I.—The use of Table I eliminates the need for calculating positions on the probability scale for each successive value in a series. For odd numbers of data less than 26, percentages found in the table may be used directly, and for even numbers the percentages chosen from the column referring to the next highest number are used with the omission of the mid-point of scale (50% division) in plotting. For numbers of values greater than 25, it has been found convenient to group the data so as to supply 25 or fewer groups. Proper grouping is necessary in

order to facilitate plotting with the aid of Table I or the automatic guide described above, and grouping errors can be minimized by selecting as large a number of groups as practicable.

In every case it is necessary to arrange the data in serial order of increasing or decreasing magnitude before grouping. Serial arrangement may be conveniently accomplished by tabulating the data in vertical columns covering the range of the values. Only the last figure of each value need be tabulated in its proper column at an appropriate relative position that provides space to arrange all values that may accumulate in the column.

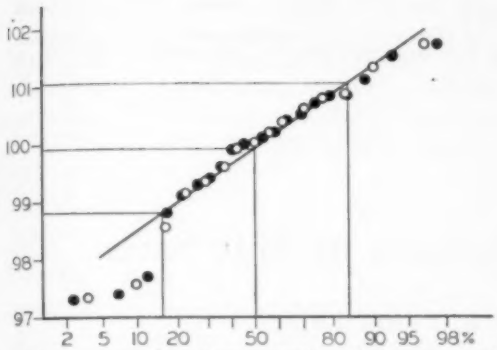

Fig. 3.—Graphical determination of the mean and standard deviation of the iodometric determinations of penicillin reported by Mundell, *et al.* (10). Open circles represent averages of 15 groups of 4 values each; dark circles represent every third value, beginning with the second, taken from the same data after serial arrangement. Per cent recovery of penicillin is plotted against reading points automatically selected by the instrument.

For the purpose of illustration, the sixty comparative assays of crystalline penicillin G by iodometric titration given by Mundell, Fischbach, and Eble (10) were selected for study. Two general methods of grouping are illustrated in Fig. 3, where the open circles represent 15 points plotted from the averages of groups of four values selected after serial arrangement. The black circles represent 20 values selected by taking every third value beginning with the second in the serially arranged list of values. It will be noted that both methods are in agreement, and that, for the most part, the data are normally distributed. The few points that fail to fall on the straight line are given little weight in fitting the line from which the mean is read as 99.93, a value closer to the theoretical 100% recovery than the value of 99.82 obtained by Mundell, *et al.*, by conventional methods. A difference between the arithmetic mean and the value of the mean obtained from the best visual fit to the straight line indicating normal distribution is to be expected if some of the values are given little weight because they do not fall on the normal distribution curve. For the same reason, values for the standard deviation are slightly smaller than those calculated in the usual way (in this case, 1.11 instead of 1.20).

Use of a Vertical Logarithmic Scale.—The calculator is so constructed that a logarithmic scale may be used interchangeably with the linear vertical scale for the purpose of studying data that are best handled by means of logarithmic probability paper (3, 5), such as the plotting of log dose of a poison against the percentage of test organisms succumbing (11, 12). A straight line, visually fitted to such a plot, permits a direct estimate of LD_{50} from the point where the line intersects the vertical log scale when the reading line of the cursor coincides with the mid-point of the probability scale. LD_{95} may also be read by setting the cursor over the 95% position on the probability scale. While this method is not as highly refined as methods developed for the use of the probit scale (8, 13), it has the advantage of simplicity and speed. A probit scale may be drawn for use as an auxiliary probability scale if desired. To construct such a scale, the reading point near the 16% position on the probability scale, described above, is probit 4; the 50% division is probit 5, and the remaining reading point (84.14%) is probit 6. Equal subdivisions and linear extensions complete the probit scale.

SUMMARY AND CONCLUSIONS

The graphical calculator provides a practical way of making use of the known mathematical properties of arithmetic probability paper in testing for deviations from normal distribution and in estimating the mean and the standard deviation from the mean. Equipped with a vertical log scale, the instrument may be used to advantage in estimating other statistics (i.e., LD_{50}). In general, the graphical calculator may be thought of as providing a rapid visual estimate of quantities that may later require more refined analysis. In testing for significance, for example, it is important to use the values obtained from the graphical calculator with the same degree of caution that would be applied to the use of the same values calculated in any other way. However, there can be no doubt that the data plotted in Fig. 3 are much more illuminating than pure analytical calculations because of the clear demonstration of their relation to normal distribution.

This example illustrates the importance of the geometrical vs. the arithmetical approach in statistical analysis, emphasised by Treloar (14) by the inclusion of the following

quotation from Francis Galton: "It is always well to retain a clear geometric view of the facts when we are dealing with statistical problems, which abound in pitfalls, easily overlooked by the unwary, while they are cantering gaily along upon their arithmetic." Rissik (3) makes the same point more directly in the statement: "The advantages attaching to the use of probability graph paper in the solution of statistical problems involving a determination of the mean and standard deviation of a series of quantitative data are so great that, in the author's opinion at any rate, the graphical method is nearly always to be preferred."

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The Solubilization of Quinine by Bile Salts*

By S. MUKHERJEE and R. P. BANERJEE†

From turbidimetric titrations of bile salts with quinine and turbidity-concentration curves of colloidal solutions of quinine-dehydrocholic acid and tauroglycocholic acid complexes, it has been concluded that solubilization takes place through the formation of micelles composed of quinine and bile salts. Two types of micelles are envisaged; primary micelles built up by the association of quinine with bile salt molecules or with very small micelles; and secondary micelles formed of quinine molecules and large micelles of bile salts. The primary micelles are easily diffusible while secondary micelles form irreversible precipitates. Solubilization is accompanied with the formation of primary micelles. This would explain the difference in solubilizing powers of sodium dehydrocholate and tauroglycocholate.

IT HAS LONG been known that bile salts can render water-soluble many water-insoluble substances, e.g., fats,, fatty acids, alcohols, ethers, etc. The mechanism by which this solubilization takes place has evoked much interest. The earliest hypothesis for explaining this phenomenon is the "choleic acid principle" of Wieland and Sorge (1), which postulates the formation of soluble co-ordination complexes of these substances with the bile salts. It appears from the recent work of McBain and co-workers (2, 3) that solubilization can also be brought about by many other detergent substances which can be classed as colloidal electrolytes. According to the hypothesis put forward by these authors, solubilization is effected by the sorption of the insoluble substance on the micelles of the detergent solution. Thus, the greater the micelle-forming ability of a detergent, the greater would be its solubilizing power. Roepke and Mason (4) have recently shown that, above a certain "critical" concentration, solutions of bile salts also contain micelles, and these can therefore be regarded as colloidal electrolytes.

Mukherjee, Banerjee, and Basu (5) have shown that bile salts can solubilize quinine under favorable conditions. When a solution of quinine salt is added gradually to an alkaline bile salt solution, a stable colloidal solution is formed, so long as the mixture remains alkaline and the molar ratio of qui-

* Received Jan. 29, 1947, from the Bengal Immunity Research Laboratory, Calcutta, India.
† The authors wish to thank Dr. U. P. Basu for his interest in this work.

nine to the bile acid does not exceed about 1:4. When such a colloidal solution is dialyzed in a bag of cellophane, swollen in 64 per cent zinc chloride solution, both the quinine and the bile acid can diffuse out into the dialysate. An investigation of the nature of this colloid and the mechanism of the solubilization appeared to be of interest.

EXPERIMENTAL

Turbidimetric titrations of sodium dehydrocholate and tauroglycocholate with quinine were carried out in the following manner: To 25 ml. of a 0.0468 *M* solution of either the dehydrocholate or the tauroglycocholate was added gradually a 0.068 *M* solution of quinine hydrochloride, and the change in the turbidity of the resulting mixture was followed using a Klett–Summerson photoelectric colorimeter. The titration curves are shown in Fig. 1.

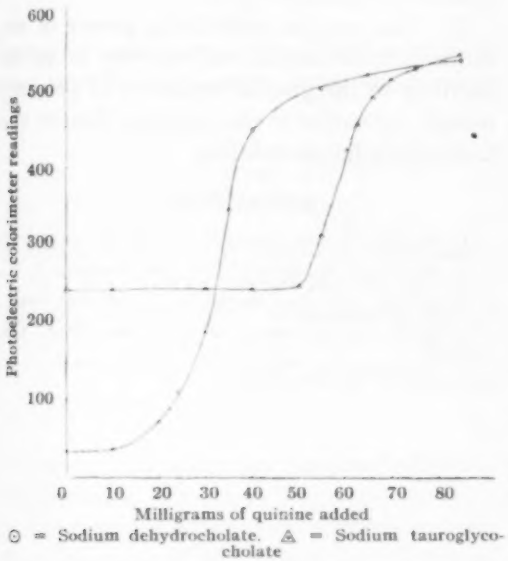

Fig. 1.—Turbidimetric titrations with quinine hydrochloride.

A study was also made of the changes of turbidity of these colloids with dilution. The respective colloidal solutions were prepared by gradually adding 8.5 ml. of a 0.068 *M* solution of quinine hydrochloride to 50 ml. of a 0.0468 *M* solution of sodium dehydrocholate or tauroglycocholate. The colloidal solutions thus obtained were diluted with water to different extents, and the diluted solutions were set aside for two hours, with frequent stirring, before taking readings in the photoelectric colorimeter. The turbidity-concentration curves have been shown in Fig. 2, the concentrations being expressed as percentages of the undiluted colloids.

The initial lag in the quinine-dehydrocholate titration curve (Fig. 1) indicates that micelle formation, leading to turbidity, takes place only after a limiting concentration of the complex is reached. The number of micelles increases with further increase in the concentration of quinine. Again, dilution of quinine-dehydrocholate colloid (Fig. 2) is attended by a

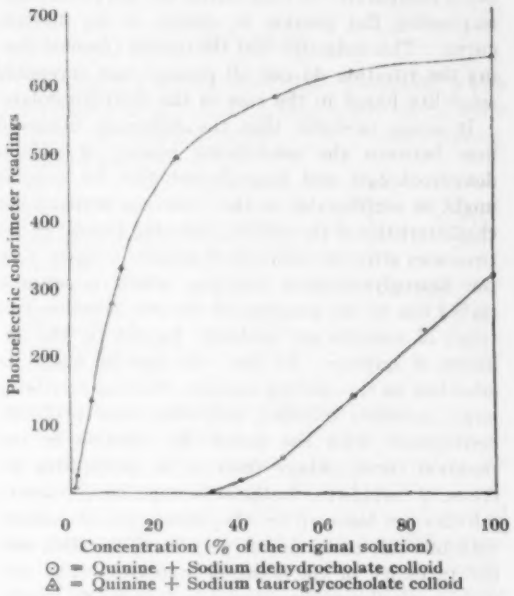

Fig. 2.—Turbidity-concentration curves of quinine + bile salt colloids.

regular, almost linear, fall in turbidity, the solution becoming practically clear and free from micelles when the concentration is about 30% of the original. These results indicate that the micelles of the quinine-dehydrocholate complex, obtained as above, possess a reversible-equilibrium solubility, as would be expected of a true colloidal electrolyte.

The observations of Mukherjee, Banerjee, and Basu (5) that, during dialysis of the colloids, quinine and bile salt diffuse out through the membrane simultaneously, and that the first day's dialysate also becomes turbid, give support to the view that the solubilization of quinine is effected by the formation of micelles (containing both quinine and the bile acid) which can penetrate the membrane.

McVain, Merrill, and Vinograd (2), working with the water-insoluble dye, Yellow AB (phenylazo-2-naphthylamine), found that sodium dehydrocholate had no solubilizing action on this dye, while sodium cholate, taurocholate, and deoxycholate possessed marked solubilizing power. They explained the difference on the basis of differences in the micelle-forming abilities of these bile salts.

Work in this laboratory (5) has shown, however, that both sodium dehydrocholate and tauroglycocholate can solubilize quinine, and that the dehydro-

cholate possesses this power in the greater degree, since, with the tauroglycocholate, a part of the quinine is thrown down as a coarse precipitate and is thus rendered nondiffusible. The titration curve for tauroglycocholate (Fig. 1) and the turbidity-concentration curve (Fig. 2) of the colloid formed from it show some characteristic differences from the corresponding curves for sodium dehydrocholate. While the titration curve for the tauroglycocholate has a comparatively long initial flat portion, a corresponding flat portion is absent in the dilution curve. This indicates that the micelles formed during the titration do not all possess that reversible solubility found in the case of the dehydrocholate.

It seems probable that the difference observed here between the solubilizing powers of sodium dehydrocholate and tauroglycocholate for quinine might be attributable to the difference between the characteristics of the colloidal micelles formed in the two cases after the addition of quinine to them. In the tauroglycocholate solution, which is initially turbid due to the presence of its own micelles, two types of micelles are probably formed on the addition of quinine. At first, the quinine would be adsorbed on the existing micelles, forming relatively large *secondary* micelles, and this stage probably corresponds with the initial flat portion in the titration curve (where there is no perceptible increase of turbidity). In the next stage, fresh *primary* micelles are built up by the association of quinine with bile salt molecules or very small micelles, and the turbidity of the solution increases. The secondary micelles might completely or partially coagulate due to large size or because of the neutralization of their changes during the adsorption of quinine, and probably do not possess the reversible solubility which is shown by the *primary* micelles. On the other hand, the absence of turbidity in the sodium dehydrocholate solution shows that only *primary* micelles are formed on the addition of quinine. Such a hypothesis would seem to fit in with the experimental results and explain the greater solubilizing power of sodium dehydrocholate for quinine.

SUMMARY AND CONCLUSIONS

1. Turbidimetric titrations of sodium dehydrocholate and tauroglycocholate with quinine salts, and turbidity-concentration curves of the quinine and bile salt colloids, show evidence of micelle formation during the interactions.

2. The micelles formed by these reactions are termed *primary* if they are freshly formed from dissolved molecules, and *secondary* if formed from micelles of bile salts already existing and in the solution. The formation of *primary* micelles is responsible for the solubilization of quinine. *Secondary* micelles are relatively unstable and lead to irreversible precipitation.

3. The greater solubilizing power of sodium dehydrocholate for quinine is to be ascribed to the greater tendency of *primary* micelle formation in this solution than in the tauroglycocholate solution.

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WHO MAKES IT?

The National Registry of RareChemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Xanthopterin
Perseitol
Perseulose
l-Erythrulose
Butoxy acetic acid
Hydrindene (1 Kg.)
Erythroidine
4-Nitro pyrocatechol
o-Tolyl urethane
Montanyl alcohol
Melissyl alcohol
Saccharic acid
Carnaubyl alcohol
Ceryl alcohol
l,d or *dl*-Pseudoephedrine or salts
l,d or *dl*-Norephedrine or salts
Stachyose
Lactosin
Phenoxazine
Dihydroscillaren A
Isodigitoxigenin
Uzarin

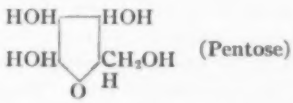
The Nitrofurans—A New Type of Antibacterial Agent*

By R. J. MAIN

This report reviews the basis upon which the present status of Nitrofurazone (Furacin) depends. This compound is either bacteriostatic or bactericidal to a wide variety of organisms and possesses a low degree of systemic and local toxicity although sensitization may sometimes be encountered.

THE ANTISEPTIC action of various furan derivatives such as furfural (R═CHO) and furoic acid (R═COOH) has been recog-

nized for some time (1–5). These substances are usually synthesized from the pentose



(Pentose)

sugars of oat hulls by chemical dehydration.

In 1944, Dodd and Stillman (6) reported that the addition of a 5-nitro group conferred

a marked increase in antibacterial power on the furans.

Over one hundred such nitrofurans were then synthesized and tested for their antibacterial potency. The most active of these were studied from such standpoints as solubility, systemic toxicity, irritation, and effect on the healing rate of experimental wounds.

The nitrofuran which finally best met the necessary requirements of a topical antibacterial agent for clinical use was nitrofurazone: 5-nitro-2-furaldehyde semicarbazone.

This was named *Furacin, a brand of nitrofurazone*, N. N. R.

FURACIN

Furacin is a yellow crystalline compound which decomposes at 227° and darkens slowly on exposure to light. Its solubility is rather low in most solvents, as shown below:

	Gm.	Cc.
Water	1	4,200
Alcohol, 95%	1	590
Acetone	1	415
Ether	1	12,500
Benzene	1	43,500
Chloroform	1	27,000
Propylene glycol	1	300
Carbowax 1500[1]	1	100

It shows good stability in such solutions.

Bacteriology.—Furacin is either bacteriostatic or bactericidal *in vitro* to many bacteria, including the majority of those commonly found in surface infections, such as wounds and burns. Its antibacterial spectrum encompasses many Gram-negative as well as Gram-positive organisms. Preliminary results (6, 7) and studies in progress indicate that Furacin may be bactericidal rather than merely bacteriostatic to the majority of these organisms. It is not fungicidal or fungistatic.

The antibacterial spectrum of Furacin in broth includes (6–8):

Gram-negative	Gram-positive
Neisseriae gonorrhoeae	*Staphylococcus aureus*
Neisseriae intracellularis (Meningococcus)	*Staphylococcus albus*
Escherichia coli	*Streptococcus faecalis*
Salmonella schottmuelleri (Paratyphi B)	*Streptococcus anhemolyticus*
Salmonella paratyphi (Paratyphi A)	*Streptococcus pyogenes* (*S. hemolyticus*)
Eberthella typhosa	*Diplococcus pneumoniae* I
Shigella dysenteriae	*Clostridiumhistolyticum*
Proteus vulgaris	*Clostridium novyi*
Chromobacterium violaceum (*Bacillus violaceus*)	*Clostridium sporogenes*
Serratia marcescens	*Clostridium tetani*
Aerobacter aerogenes	*Clostridium septicum*
Klebsiella pneumoniae (Friedlander's bacillus)	*Clostridium perfringens* (*B. welchii*)
	Mycobacterium tuberculosis var. *hominis*
	Mycobacterium tuberculosis Gary
	Bacillus subtilis
	Bacillus anthracis
	Corynebacterium diphtheriae

Furacin exerts its antibacterial effects in broth at rather low concentrations, which vary with different

* Received May 17, 1947, from the Eaton Laboratories, Norwich, N. Y.

[1] A brand of polyethylene glycol.

organisms. Bacteriostatic action within twenty-four hours is obtained against most of the above organisms at concentrations between 1 to 200,000 and 1 to 20,000. In comparison, the concentration of Furacin as used clinically in Furacin Soluble Dressing is 1 to 500.

Fig. 1.—Furacin Crystal.

Determination of the extent of the antibacterial spectrum is complicated by the fact that some strains of the above organisms prove to be resistant *in vivo*. On the other hand, certain bacteria not regularly inhibited by Furacin in broth cultures, such as *Pseudomonas pyocyaneus*, have been successfully combatted in clinical infections (9) and are often susceptible to Furacin Soluble Dressing in agar cup-plate tests. This may result from the greater solubility of Furacin in the base used, as compared to its solubility in purely aqueous solutions.

Laboratory and clinical studies have not yet reached a point where any statement can be made concerning possible development of fastness of bacteria to this agent. Judging from the existence of this phenomenon with other modern antibacterial agents, we may expect comparable effects with Furacin. Green and Mudd (10) have shown that development of resistance by various bacteria to penicillin, streptomycin, and sulfathiazole does not affect their susceptibility to Furacin *in vitro*.

Furacin retains its antibacterial power *in vitro* in the presence of blood, serum, large bacterial innocula and broth, although its minimal effective concentrations must then be increased from 2 to 10 times (6, 7, 11). It has been shown to be effective by oral administration in systemic infections of mice with several types of bacteria and *Trypanosoma equiperdum* (11).

When applied topically, it is capable of diminishing infection with Streptococcus hemolyticus of experimental rabbit wounds (12). It has also been successful in reducing the infection of human wounds and burns (9, 13, 14) as shown by bacterial cultures from the lesions. Such evidence indicates that its antibacterial power *in vivo* is not seriously inhibited by the presence of organic material such as is found in wounds.

The mode of action of Furacin on bacteria is still unexplained. Cramer and Dodd (7) suggested that this nitrofuran may affect adversely some metabolic function of the bacterial cell, perhaps the oxidation-reduction system. They found that, at low concentrations of Furacin, the lag phase of bacterial growth was prolonged, rather than the rate of reproduction being diminished once it was initiated. Krantz and Evans (15) proved that Furacin is capable of diminishing the activity of tissue dehydrogenases.

Toxicity.—The toxicity of Furacin is relatively low, for that was an important factor in choosing this particular nitrofuran for clinical use. Krantz and Evans (15) report the oral LD_{50} for rats as 0.59 Gm. per Kg., which is identical with the dose for mice (11). These investigators fed rats a diet containing 0.2% Furacin, for five weeks without demonstrable ill effects, but 0.4% proved fatal. Monkeys fed 0.3 Gm. daily for five weeks remained normal. Human volunteers have ingested as much as 4 Gm. daily without ill effects, except for nausea in some subjects. Furacin had no demonstrable acute effects when injected intravenously into dogs, cats, and monkeys. For this purpose the following solutions and amounts were used: a saturated solution of Furacin in propylene glycol (1:350), administering 0.5 cc. per Kg., and 0.5% or 1% suspensions of Furacin in 6% aqueous acacia solution, using 0.5 cc. or 1 cc. per Kg.

Since Furacin is being used only in the form of a 0.2% ointment, no toxic effects from absorption would be expected, nor have any been reported from the clinic. This expectation is further substantiated by the investigation of Dodd, Hartmann, and Ward (16), who applied Furacin to large cutaneous wounds of rabbits. A dose of 0.25 Gm. per Kg. was used, dissolved in 2 Gm. of the carbowax-propylene glycol base. The wound was kept covered and reapplication of the drug was made at weekly intervals, for four weeks. There were no effects on weight, blood pressure, or blood urea.

Furacin apparently has little cytotoxicity, for Furacin Soluble Dressing does not retard the healing rate of experimental wounds (16). Clinical investigators also report that use of Furacin Soluble Dressing does not appear to delay granulation or epithelization (9, 14).

Sensitization.—In common with other topical antibacterials, Furacin, in the form of Furacin

Soluble Dressing, produces sensitization phenomena in certain susceptible patients. Preliminary reports indicate that this occurs infrequently. Downing, Hanson, and Lamb (13) found such effects in 8 of 212 patients. Shipley and Dodd (9) report it in 2 of 90 patients. The reaction consists of a red, macular, or papular rash which may be generalized or restricted to the area in contact with the preparation. This eruption usually disappears within a few days after cessation of therapy, but may last longer.

Constant application of Furacin, in Furacin Soluble Dressing, to the skin of 10 normal subjects for two days produced no effects (16). Downing, *et al.* (13) tested 200 normal subjects, in comparable fashion for five days, and found mild erythema at the end of two days in 5.5% of the subjects. This effect disappeared within five days. Fifty of these subjects were retested in ten days, with only 4% showing erythema, indicating absence of sensitization in this group.

FURACIN SOLUBLE DRESSING

Furacin has recently been made available to the medical profession, first in the form of Furacin Soluble Dressing, a topical antibacterial agent for the treatment of wound and surface infections. Its use does not replace necessary surgical intervention such as debridement and drainage. This preparation is accepted by the Council on Pharmacy and Chemistry of the American Medical Association. It contains Furacin 0.2% (brand of nitrofurazone) dissolved in a water-soluble base composed of a mixture of glycols. These ingredients of the base are innocuous and acceptable to the Food and Drug Administration for topical use (17–21).

Furacin Soluble Dressing liquefies at body temperature, forming a clear, thin, surface-active liquid which can penetrate small crevices. Its clarity enables the clinician to inspect the wound without removing the drug. However, when removal is desired, its water-solubility permits its ready removal by simple irrigation with water or saline. Such water-solubility also permits it to dissolve in pockets of wound exudates which may persist in spite of drainage. This helps the active ingredient to reach all infected areas. The compound cannot dry or cake, to interfere with wound drainage. The light-yellow color is readily removed from linens by washing. Its *p*H lies between 5 and 7.

Clinical Reports.—The first published clinical article on Furacin was by Snyder, Kiehn and Christopherson (14), who used Furacin Soluble Dressing on infected war wounds and burns incurred during the Normandy invasion. They reported their results on 10 patients, most of whose wounds were infected by several different bacteria. They concluded that topical Furacin therapy appeared to be very effective, as evidenced both by rapid improvement and reduction of the bacterial flora.

Downing, Hanson, and Lamb (13) recently reported their results on 212 patients with various dermatological infections, such as infectious eczematoid dermatitis, impetigo, and infected hypostatic (varicose) ulcers. They state that this drug shows excellent possibilities in the treatment of superficial infections of the skin, producing a high percentage of control of both Gram-positive and Gram-negative bacteria.

Shipley and Dodd (9) used this new antibacterial agent on 90 patients with various infections, such as burns, abscesses, ulcers, and wounds. Of these, they obtained good results in 70 cases. Discharge and odor of the wounds were lessened in the great majority of the cases. Of the strains of the common wound bacteria isolated from these patients, 80% were reduced or became negative during treatment, and 20% were not affected.

Indications.—From these results and those being obtained in other investigations now in progress, Furacin Soluble Dressing is suggested as an aid in the treatment of the following conditions. It is not a substitute for surgical treatment.

Infected surface wounds, or for the prevention of such infection;
Infections of second and third degree burns;
Carbuncles and abscesses after surgical intervention;
Infected varicose ulcers;
Infected superficial ulcers of diabetics;
Impetigo of infants and adults;
Treatment of skin-graft sites;
Osteomyelitis associated with compound fractures;
Secondary infections following dermatophytoses.

Dispensing.—Furacin Soluble Dressing is supplied in 1-lb. jars and 4-oz. jars. In filling prescriptions for smaller amounts, the pharmacist should use opal or amber ointment jars. Some metals may cause Furacin to darken slowly.

Because Furacin Soluble Dressing darkens on prolonged exposure to light, the jar should be kept covered. Such darkening is a surface phenomenon and does not measurably diminish its antibacterial efficiency. The preparation is stable at room temperature; no refrigeration is required.

Modes of Application.—Clinicians apply Furacin Soluble Dressing in various ways. Some place it directly on the wound. Others apply it to a gauze dressing which is then laid on. For applying it to large areas, as for severe burns, it may be spread over large strips of gauze, which are then laid on top of each other in a sterile tray. This is heated to melt the compound so that it impregnates the gauze thoroughly. The preparation is stable at autoclave temperatures, for at least fifteen minutes. When these strips are applied to the patient, petrolatum-impregnated gauze or some other impermeable dressing may be laid on top, to prevent the Furacin Soluble Dressing from being absorbed by the bandage, with consequent adhesion of the bandage to the wound by dried wound exudates.

Because this preparation darkens on exposure to light, it is usually advisable to cover it with a gauze

dressing, especially when used on exposed areas, as for impetigo.

Reapplication of Furacin Soluble Dressing may be made as often as four times daily for such conditions as impetigo, to once or twice daily, or even less often, for wounds and burns.

SUMMARY

The antibacterial power of compounds containing the furan nucleus is markedly increased by addition of a 5-nitro group.

Nitrofurazone (Furacin) is the first nitrofuran to be made available for clinical use. It is either bacteriostatic or bactericidal to a wide range of organisms, including both Gram-negative and Gram-positive bacteria, both *in vitro* and *in vivo*. It has low systemic and local toxicity. Sensitization may be encountered.

Furacin is now available as Furacin Soluble Dressing for the topical treatment of wound and surface infections.

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Book Review

Bacteriology—Laboratory Directions for Pharmacy Students, Second Edition. Edited by MILAN NOVAK and ESTHER MEYER. C. V. Mosby Company, St. Louis, Mo., 1947. 248 pp. 26.5 x 19 cm. Price $2.75.

This outline was designed to present a systematic introduction of laboratory procedures to be used by beginning students in the study of bacteriology. Since time is an important factor in dealing with living things, it is imperative that the schedule used in the daily study of microorganisms be carefully planned in advance. This is particularly true when the work of two or more days must overlap due to the slow rate of multiplication of many bacterial and mold species.

This book presents detailed instructions of techniques to be followed throughout an entire laboratory course in bacteriology. In all, thirty-six sections, or periods, covering the various phases of handling bacteria, their significance in infections, differential diagnostic methods for identifying bacteria by stains, media, serology, etc., are presented in exacting details. Consideration is also given to the more recent advancements made in bacteriology to include the effects of sulfa drugs, antibiotics, and antiseptics on microorganisms. Details and interpretations of test procedures involving sterility testing of pharmaceutical preparations, fungicidal and fungistatic tests on ointments, powders and liquids as well as microbiology of food and pharmaceutical syrups are timely subjects which should prove to be of valuable interest to pharmacy students.

While the outline may be considered a complete presentation of laboratory methods, the reviewer questions the desirability of including discussions of various phases of bacteriology in reference to theories, historical development, definitions, etc. These points are often interspaced in the daily outline of laboratory directions, thereby requiring time to read and pick out the essentials assigned for the day's program. Since reference is made in the introductory chapter to the effect that the laboratory work is to be regarded as a supplement to regular didactic lectures and frequent demonstrations, it would appear more desirable to discuss the general principles of bacteriology at these meetings.

It is also anticipated that each student will personally have the opportunity to prepare at least one bacteriological medium throughout. In addition, the principles of sterilizing by autoclaving, dry heat, or filtration can only be impressed upon the student by his own participation in these procedures.

In general, the outline is a complete one and should prove to be a valuable aid to the teaching of laboratory bacteriology, not only to pharmacy students, but to medical students as well. A novel feature of the manual is the modern loose-leaf plastic binding which permits removal or reinsertion of pages when necessary.—C. A. LAWRENCE.